Regulatory Polymorphisms in β-Tubulin IIa Are Associated with Paclitaxel-Induced Peripheral Neuropathy

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Abstract

Purpose: Peripheral neuropathy is the dose-limiting toxicity of paclitaxel, a chemotherapeutic drug widely used to treat several solid tumors such as breast, lung, and ovary. The cytotoxic effect of paclitaxel is mediated through β-tubulin binding in the cellular microtubules. In this study, we investigated the association between paclitaxel neurotoxicity risk and regulatory genetic variants in β-tubulin genes.

Experimental Design: We measured variation in gene expression of three β-tubulin isotypes (I, IVb, and IIa) in lymphocytes from 100 healthy volunteers, sequenced the promoter region to identify polymorphisms putatively influencing gene expression and assessed the transcription rate of the identified variants using luciferase assays. To determine whether the identified regulatory polymorphisms were associated with paclitaxel neurotoxicity, we genotyped them in 214 patients treated with paclitaxel. In addition, paclitaxel-induced cytotoxicity in lymphoblastoid cell lines was compared with β-tubulin expression as measured by Affymetrix exon array.

Results: We found a 63-fold variation in β-tubulin IIa gene (*TUBB2A*) mRNA content and three polymorphisms located at -101, -112, and -157 in *TUBB2A* promoter correlated with increased mRNA levels. The -101 and -112 variants, in total linkage disequilibrium, conferred *TUBB2A* increased transcription rate. Furthermore, these variants protected from paclitaxel-induced peripheral neuropathy [HR, 0.62; 95% confidence interval (CI), 0.42–0.93; $P = 0.021$, multivariable analysis]. In addition, an inverse correlation between *TUBB2A* and paclitaxel-induced apoptosis ($P = 0.001$) in lymphoblastoid cell lines further supported that higher *TUBB2A* gene expression conferred lower paclitaxel sensitivity.

Conclusions: This is the first study showing that paclitaxel neuropathy risk is influenced by polymorphisms regulating the expression of a β-tubulin gene. *Clin Cancer Res; 18(16); 4441–8. ©2012 AACR.*

Introduction

Paclitaxel is a microtubule-binding drug widely used for the treatment of several solid tumors, such as breast, ovary, and lung (1). Paclitaxel binds the β-subunit of the tubulin dimers, the main components of cellular microtubules (2), leading to their stabilization, cell-cycle block, and cell death (3, 4). The current paclitaxel dose-limiting toxicity is peripheral neuropathy (5, 6), which is predominantly sensory, and develops as a painful, debilitating, and symmetrical distal axonal neuropathy (7, 8). Although the mechanisms causing this toxicity have not been precisely determined, it is clear that the microtubule-mediated axonal transport is affected (9–11). Paclitaxel neurotoxicity is dose-cumulative, with some clinical factors influencing toxicity risk (12, 13). However, a large part of the interindividual variability remains unexplained, and whereas some patients are asymptomatic, others have to discontinue paclitaxel treatment due to the neuropathy. The symptoms usually disappear over months after paclitaxel treatment is stopped, but severe cases can have irreversible peripheral axonal damage. Our group and others have investigated the contribution of genetic variation in paclitaxel pharmacokinetic pathway to neurotoxicity risk (14, 15); however, a large part of paclitaxel-induced neurotoxicity variability remains unexplained.

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Translational Relevance

Paclitaxel is a microtubule-binding drug widely used to treat several solid tumors, such as breast, ovary, and lung. The current paclitaxel dose-limiting toxicity is peripheral neuropathy, which is dose-cumulative and occurs in about one third of the patients. It exhibits a large interindividual variability of unknown molecular basis, with some patients asymptomatic whereas others discontinue paclitaxel treatment due to the neuropathy, with severe cases with irreversible peripheral axonal damage. In this study, we provide novel insights into the biology underlying paclitaxel neurotoxicity interindividual variability by using different cell line models and an outstanding series of 214 well-characterized patients treated with paclitaxel. We identified two common regulatory polymorphisms in the proximal promoter of β-tubulin IIa, the therapeutic target of paclitaxel, that confer an increased transcription rate and protect from paclitaxel-induced peripheral neuropathy [HR, 0.62; 95% confidence interval (CI), 0.42–0.93; $P = 0.021$, multivariable analysis]. These variants could provide the basis for an individualized paclitaxel pharmacotherapy.

Although neuron β-tubulins are the therapeutic target that mediates paclitaxel neurotoxicity, these molecules have not been investigated in relation to the neuropathy. We have previously shown that neuronal microtubules are formed by 6 different isotypes: IVa, IIa, IVb, IIb, I, and III, with β-tubulin IVa and IIa being the majority forms and constituting more than 75% of the total β-tubulin content in brain (16). This tissue contains the highest amounts of β-tubulin, probably reflecting the importance of the extensive neuronal cytoskeleton for the diverse functions of the human neurons. β-Tubulin I and IVb are ubiquitous isotypes, isotype IIa has a broad expression, whereas the expression of β-tubulin IIb, III, and IVa is mainly restricted to neurons (16).

β-Tubulins are highly conserved proteins, and polymorphisms leading to amino acid changes have been ruled out for all isotypes except for the hematologic-specific β-tubulin VI (ref. 17; Leandro-García et al., submitted for publication). In fact, missense variants in the neuron-specific β-tubulins IIb and III are pathogenic and lead to a spectrum of severe neuronal disorders (18, 19). Concerning variations in gene expression, β-tubulin III has been found overexpressed in tumors, and this event has been associated with poor prognosis and altered drug response in various tumor types (20–22). However, constitutive variability in the expression of these isotypes due to regulatory polymorphisms has not been investigated.

In this study, we show that there is a large interindividual variability in β-tubulin IIa mRNA expression and that 2 genetic variants in total linkage disequilibrium in the promoter region of the β-tubulin IIa gene (*TUBB2A*) are

involved in this variation. Furthermore, genotyping of 214 patients treated with paclitaxel showed that these polymorphisms are associated with paclitaxel neuropathy risk. In addition, an association between paclitaxel-induced apoptosis and β-tubulin IIa expression was further confirmed using cell lines.

Materials and Methods

Human biological samples

Lymphocytes were isolated from total peripheral blood samples from 100 healthy volunteers by density-gradient separation in Histopaque-1077 (Sigma-Aldrich) as previously described (23). DNAs from 214 patients with cancer treated with paclitaxel were collected with the collaboration of 1 Spanish and 2 Swedish centers: 118 patients corresponded to the Hospital Universitario Fundación Alcorcón (Madrid, Spain; ref. 15), 63 to the Karolinska Institutet (Stockholm, Sweden), and 33 to the Linköping University (Linköping, Sweden; ref. 24). Ovary, lung, and breast cancer were the most common malignancies from the patients, grade III neurotoxicity was observed in 11% of the patients and grade II in 39%. Patient characteristics, chemotherapy regimens, and neurotoxicity data are summarized in Table 1. The collection of samples was approved by the corresponding Internal Ethical Review Committee, and all patients signed a written informed consent before the collection of a blood or saliva sample.

RNA isolation and quantitative reverse transcription PCR

RNA was extracted from lymphocytes using TRI reagent (Molecular Research Center Inc.) and the concentration quantified by using NanoDrop ND-1000. One microgram of total RNA was reverse-transcribed using Superscript II (Invitrogen) and an oligo(dT)$_{14}$ primer following the manufacturer's instructions. The mRNA content of the different β-tubulin isotypes was quantified by quantitative reverse transcription PCR (RT-PCR) with the Sequence Detection System 7900HT (Applied Biosystems) using conditions, primers, and probes previously described (ref. 16; Supplementary Table S1). Normalization was carried out with the internal standard β-glucuronidase (*GUS*). Negative controls were included in all PCR series, and assays were carried out in triplicates. The $\Delta\Delta C_t$ method was used for the calculation of mRNA content (25).

DNA isolation, sequencing, and genotyping

Genomic DNA from lymphocytes was isolated using the FlexiGene DNA Kit (Qiagen). DNA concentration was determined using PicoGreen dsDNA quantification reagent (Invitrogen). For sequencing, *TUBB2A* promoter region was amplified by PCR using specific primers (Supplementary Table S1). PCR amplification products were purified using the PCR Purification Kit (Qiagen) and run on an ABI PRISM 3700 DNA Analyzer capillary sequencer (Applied Biosystems). Genotyping for *TUBB2A* polymorphisms located at −112 A>G (rs909965) and −157 A>G (rs9501929) was

Table 1. Characteristics of the 214 patients included in the study.

Characteristics	*n* (%)
Age at study entry, y	
Median	62
IQR (minimum–maximum)	69–56 (29—87)
Gender	
Male	42 (20)
Female	172 (80)
Site of primary tumor	
Ovary	120 (56)
Lung	39 (18)
Breast	38 (18)
Other[a]	17 (8)
Chemotherapy[b]	
Paclitaxel 175 + carboplatin	159 (74)
Paclitaxel 80	25 (12)
Paclitaxel 150 + gemcitabine	7 (3)
Paclitaxel 90 + bevacizumab	5 (2)
Paclitaxel 80 + carboplatin	5 (2)
Paclitaxel 80 + carboplatin + trastuzumab	4 (2)
Paclitaxel 175 + cisplatin	3 (1)
Paclitaxel 80 + cetuximab	2 (1)
Paclitaxel 80 + trastuzumab	2 (1)
Paclitaxel 175 + lapatinib	1 (0.4)
FAC–FEC followed by paclitaxel 80	1 (0.4)
Neurotoxicity[c]	
Grade 0	61 (28)
Grade I	46 (21)
Grade II	83 (39)
Grade III	24 (11)
Treatment modification[d]	
No change	167 (78)
Reduction	22 (10)
Suspension	25 (12)

Abbreviations: FAC, 5-fluorouracil, Adriamycin, cyclophosphamide; FEC, 5-fluorouracil, epirubicin, cyclophosphamide; IQR, interquartile range.

[a]Other sites of primary tumor were uterus, head and neck, bladder, urinary tract, germinal, and peritoneal.

[b]Paclitaxel 80 to 90 mg/m^2 had mainly 1-hour infusion and 150 to 175 mg/m^2 mainly 3-hours infusion. All doses in mg/m^2, if not specified otherwise. The different treatments consisted of: paclitaxel 175 + carboplatin [paclitaxel 175; carboplatin area under curve (AUC) 6/3 weeks]; paclitaxel 80 (paclitaxel 80/weekly); paclitaxel 150 + gemcitabine (paclitaxel 150; gemcitabine 2,500/2 weeks); paclitaxel 90 + bevacizumab (paclitaxel 1°, 8° and 15° day; bevacizumab 10 mg/kg 1° and 15° day/ 4 weeks); paclitaxel 80 + carboplatin (paclitaxel 80 + carboplatin AUC 2/weekly); paclitaxel 80 + carboplatin + trastuzumab (paclitaxel 80; carboplatin AUC 2; and trastuzumab 2 mg/kg/weekly); paclitaxel 175 + cisplatin (paclitaxel 175; cisplatin 90/3 weeks); in 1 patient

conducted in duplicates with the KASPar SNP Genotyping System (Kbiosciences) using 15 ng of genomic DNA. All assays included DNA samples with known genotypes and negative controls. The sequence Detection System 7900HT (Applied Biosystems) was used for fluorescence detection and allele assignment.

TUBB2A promoter cloning, transient transfection, and luciferase assay

We amplified the promoter region of β-tubulin isotype IIa gene (−389 to −15, nucleotide positions referring to *TUBB2A* translation start site ATG, +1) using specific primers that introduced *Xho*I and *Hin*dIII cleavage sites (Supplementary Table S1). The PCR product was cloned into the promoter-less pGL3-Basic Firefly luciferase reporter vector (Promega) to generate pGL3B_WT plasmid. Mutagenesis was conducted in DNA Express Inc. to generate a plasmid with −101C (rs909964) and −112G (rs909965) nucleotide changes in the promoter region of *TUBB2A* (pGL3B_−101C/−112G) and another plasmid with −157G (rs9501929) nucleotide change (pGL3B_−157G). The sequence of all the constructs was verified by DNA sequencing.

H1299 cells, derived from non–small cell lung cancer, were plated in 24-well plates and were transiently transfected with 0.3 μg of pGL3-Basic vector (EV) or the appropriate reporter constructs (pGL3B_WT, pGL3B_−101C/−112G, and pGL3B_−157G) and the internal reference *Renilla* plasmid pRL-SV40 (Promega), using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were harvested 48 hours after transfection, and lysates were used to measure firefly and *Renilla* luciferase activities using the Dual Luciferase Reporter Assay System (Promega) in a Synergy 4 Hybrid Microplate Reader (Biotek). Three independent experiments were carried out using triplicates.

Paclitaxel-induced apoptosis in lymphoblastoid cell lines

HapMap lymphoblastoid cell lines from a population with Northern and Western European ancestry from UT (HAPMAPPT01, CEU, *n* = 77) were treated with 12.5 nmol/L paclitaxel, and apoptosis (caspase-3 and -7 activity) was measured 24 hours after drug treatment using the Caspase-Glo 3/7 Assay (Promega; ref. 26). Gene expression

paclitaxel was administered intraperitoneally; paclitaxel 80 + cetuximab (paclitaxel 80; cetuximab 250/weekly); paclitaxel 80 + trastuzumab (paclitaxel 80; trastuzumab 2 mg/kg/weekly); paclitaxel 175 + lapatinib (paclitaxel 175/3 weeks; lapatinib 1,250 mg per day); and FAC–FEC followed by paclitaxel 80 (FAC/FEC followed by paclitaxel 80/weekly).

[c]Maximum neurotoxicity according to National Cancer Institute (NCI) Common Toxicity Criteria version 2.

[d]Modifications of the treatment because of paclitaxel-induced neurotoxicity.

data for *TUBB2A* in this population came from a previously published Affymetrix exon microarray analysis (27). A general linear model was constructed to test for association between log_2-transformed *TUBB2A* expression and log_2-transformed paclitaxel-induced caspase activity. A Toeplitz covariance structure with 2 diagonal bands was used to allow for familial dependencies in the data as described previously (28).

Statistical analysis

Statistical analyses were carried out using SPSS software package version 17.0 (SPSS). The method of Kolmogorov–Smirnov was used to test for normality. The Mann–Whitney nonparametric statistical test was applied to compare median β-tubulin IIa mRNA expression content. Associations between genotypes and paclitaxel neurotoxicity risk were tested using Cox regression analysis, modeling the cumulative dose of paclitaxel up to the development of grade II neurotoxicity. Patients with no or minimal adverse reaction (grade 0/I) were censored at total cumulative dose. Multivariable analysis was conducted including relevant clinical factors as covariates. Paired *t* test was used to compare the normalized luciferase activity (firefly/*Renilla*) of the different constructs. Differences were considered significant when *P* values were <0.05.

Results

β-tubulin IIa shows large interindividual differences in expression related to polymorphisms in the promoter region

We previously showed that 6 β-tubulin isotypes (IVa, IIa, IVb, IIb, I, and III) are expressed in neurons (16). Among these isotypes, IIa, IVb, and I are expressed in a wide number of tissues, including peripheral blood leukocytes, where their mRNA expression can be easily and accurately measured through quantitative RT-PCR. Thus, we quantified the expression of these 3 isotypes in leukocytes from 100 healthy volunteers. We found that β-tubulin IIa mRNA content was subjected to a large interindividual variability, 63-fold variation in expression (Fig. 1A) whereas β-tubulins IVb and I showed a 2.5- and 2.2-fold variation in mRNA content, respectively (data not shown). Variation in β-tubulin IIa expression was also found at protein level, in concordance with mRNA contents (Supplementary Fig. S1).

To investigate whether this interindividual variability in β-tubulin IIa mRNA expression could be due to genetic variability in the promoter region of *TUBB2A* gene, we sequenced the proximal promoter of the gene (300 bp) in individuals with high and low expression levels [>10,000 and <2,500 *TUBB2A* mRNA (r.u.) in $n = 9$ and $n = 11$ samples, respectively; Supplementary Table S2]. Taking into account the differences between high and low *TUBB2A* expression groups, and the linkage disequilibrium between variants, we selected −101T>C, −112A>G, and −157A>G variants (corresponding to rs909964, rs909965, and rs9501929, respectively) as potentially associated with higher *TUBB2A* expression. The minor allele frequencies of these polymorphisms in Caucasian population are 0.28, 0.28, and 0.05, respectively (http:\\www.1000genomes.org\). We found total linkage disequilibrium between −101T>C and −112A>G polymorphisms, whereas −157A>G was independent from the other 2 ($r^2 < 0.001$) and in high linkage disequilibrium with −91 G>A (rs13219681; $r^2 = 0.72$).

To elucidate whether these polymorphisms could be affecting β-tubulin IIa mRNA expression levels, we genotyped −112A>G and −157A>G in the 100 peripheral
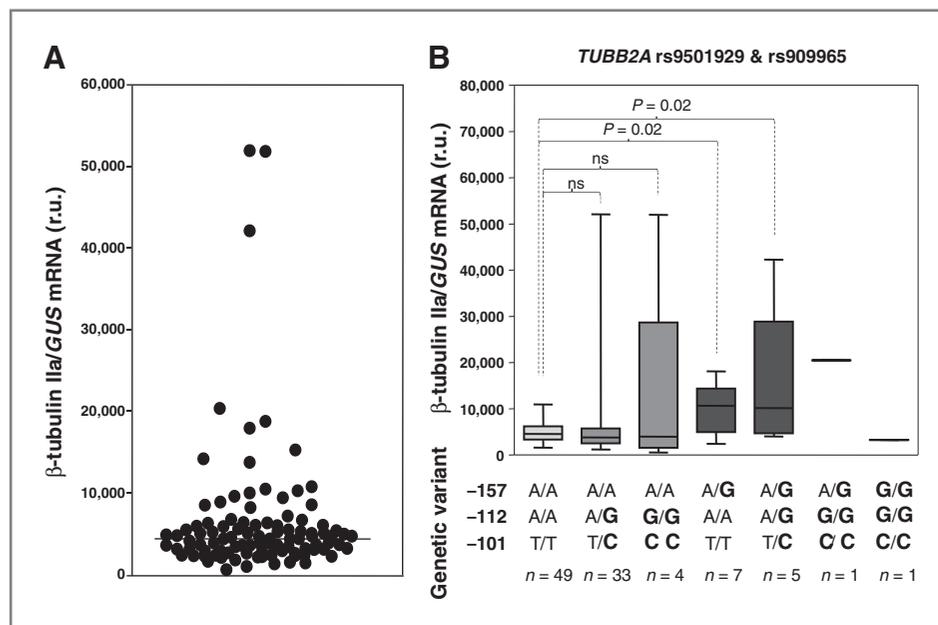
Figure 1. Interindividual variability in *TUBB2A* expression. A, *TUBB2A* mRNA content was measured by quantitative RT-PCR in 100 peripheral blood leukocytes from healthy donors, as described in Materials and Methods. The horizontal bar represents the median value. B, the healthy donors were genotyped and grouped according to the polymorphisms located at −101, −112, and −157 in *TUBB2A* promoter region (rs909964, rs909965, and rs9501929). For each genetic group, β-tubulin IIa mRNA content is represented in a box plot. The boxes show the interquartile range, the horizontal line represents the median value for each group, and the whiskers extend to the minimum and maximum values. All nucleotide positions refer to *TUBB2A* translation start site (ATG, +1).

blood lymphocytes previously used to measure mRNA expression (Fig. 1B). Lymphocytes carrying the −157G variant showed a significantly higher *TUBB2A* mRNA content ($P = 0.02$). All the remaining β-tubulin IIa high expressers were carrying the −101T/−112G variants, although the differences did not reach statistically significance. Lymphocytes simultaneously carrying −157G and −101C/−112G variants showed a significantly higher expression than the wild-type group ($P = 0.02$).

TUBB2A −101C/−112G promoter variants show an increased transcription rate in luciferase assays

To determine whether the identified *TUBB2A* promoter variants had an effect on transcription rate, we determined the transcriptional capacity of the variant promoters by transfecting the pGL3-Basic vector, pGL3B_WT, pGL3B_−101C/−112G, and pGL3B_−157G plasmids into H1299 cells. The transcriptional activity of the promoter variants measured by luciferase assay was significantly higher for the −101C/−112G variant promoter than wild-type and −157G variant promoters ($P = 0.011$ and $P = 0.018$, respectively; Fig. 2). No differences in transcriptional activity were found between −157G and wild-type promoter.

Paclitaxel neurotoxicity risk is decreased in −101C/ −112G carrier patients

Patients with cancer treated with paclitaxel were genotyped for *TUBB2A* −101C/−112G and −157G polymorphisms, and the genotypes were compared with the sensory
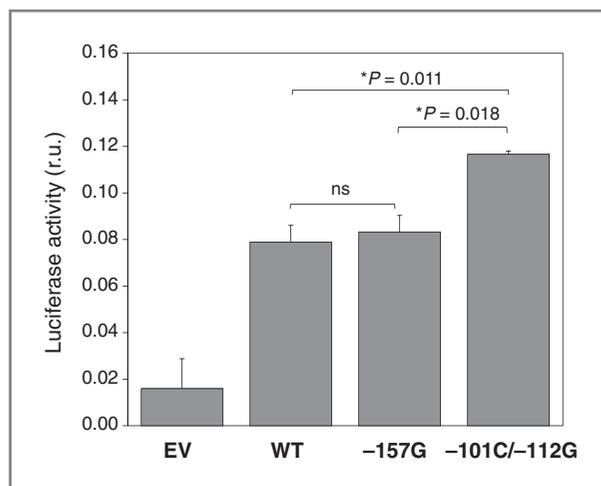
Figure 2. Effect of *TUBB2A* promoter variants on transcriptional activity. H1299 cells were transfected with pGL3-Basic (EV) and luciferase reporter plasmids with different polymorphisms in *TUBB2A* gene: pGL3B_WT (WT), pGL3B_−157G (−157G), and pGL3B_−101C/−112G (−101C/−112G). The cells were cotransfected with the pRL-SV40 plasmid containing the *Renilla* luciferase gene, which served as internal control of transfection efficiency. Promoter activities were calculated as the firefly/*Renilla* signal ratios. Mean values with SDs for the entire data set (3 transfections, each with 3 replicates) are shown. Paired *t* test was used to test differences between the luciferase activities (*P* values are shown). ns, not significant.
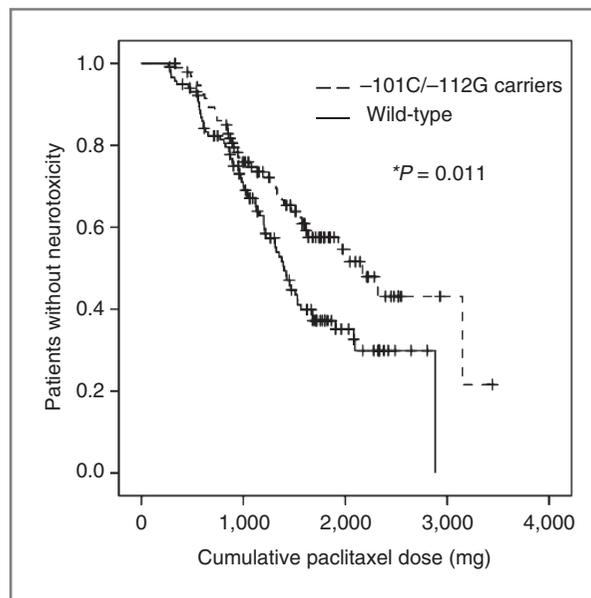
**Figure 3.** Kaplan–Meier analysis of cumulative dose of paclitaxel up to the development of grade 2 sensory peripheral neuropathy, according to −101C/−112G variants in *TUBB2A*. Patients treated with paclitaxel were grouped according to *TUBB2A* genotype. Those carrying 1 or 2 variant alleles had a significantly lower risk of paclitaxel-induced neurotoxicity. The *P* value shown corresponds to univariable log-rank test.

peripheral neuropathy developed by the patients. As shown in Fig. 3, we found that patients carrying the −101C/−112G variants had a significantly decreased risk of developing paclitaxel neurotoxicity, with an estimated HR of 0.60 [95% confidence interval (CI), 0.41–0.90; $P = 0.012$]. We confirmed that treatment schedule was an important covariate, with 80 to 90 mg/m^2 weekly scheme being more neurotoxic than 150 to 175 mg/m^2 every 21 days (HR, 1.91; 95% CI, 1.22–3.00; $P = 0.005$; ref. 29), thus, we included paclitaxel schedule as a covariate in a multivariable analysis. *TUBB2A* −101C/−112G variants showed a similar association with neuropathy protection in a Cox regression analysis adjusting for treatment schedule (HR, 0.62; 95% CI, 0.42–0.93; $P = 0.021$). When we analyzed *TUBB2A* −157G variant, we did not find statistically significant differences in paclitaxel neurotoxicity in the patients.

Increased *TUBB2A* expression is associated with decreased paclitaxel-induced apoptosis

Previously, we evaluated paclitaxel-induced apoptosis as measured by caspase-3/7 activation in 77 CEU lymphoblastoid cell lines from the International HapMap Project (30). *TUBB2A* expression was determined in the same lymphoblastoid cell lines using Affymetrix exon expression array as described previously (27). To determine whether *TUBB2A* expression and paclitaxel cytotoxic activity could be related, we compared the expression of this gene with paclitaxel-induced apoptosis. A statistically significant inverse correlation between *TUBB2A* gene expression measured and paclitaxel-induced apoptosis was found
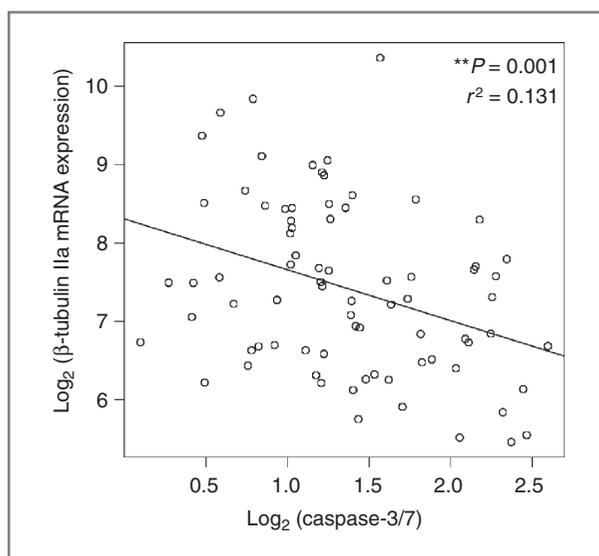
Figure 4. Inverse correlation between *TUBB2A* gene expression and paclitaxel-induced apoptosis. Lymphoblastic cell lines (CEU, $n = 77$) were treated with paclitaxel to measure caspase-3/7 activation (apoptosis), and Affymetrix exon array was used to measure *TUBB2A* expression in the same cell lines, as described in Materials and Methods. The graph shows a linear model comparing $\log_2$-transformed *TUBB2A* expression and log2-transformed paclitaxel-induced caspase activity.

($P = 0.001$; Fig. 4). This indicates that higher *TUBB2A* gene expression confers resistance to paclitaxel-induced apoptosis.

Discussion

In this work, we found a large interindividual variability in the expression of β-tubulin IIa. This isotype forms part of the neuronal microtubules, which are the therapeutic target of paclitaxel in neurons. Thus, we hypothesized that variation in β-tubulin IIa expression could be explained by regulatory polymorphisms in the promoter region of this gene and that these could contribute to the differences in toxicity observed in patients treated with paclitaxel. Specifically, 2 polymorphisms in linkage disequilibrium, −101T > C and −112A > G, showed an increased transcription rate in luciferase functional assays. Furthermore, patients carrying *TUBB2A* −101C/−112G promoter variants had a significantly reduced risk of developing neuropathy during paclitaxel treatment. The correlation between higher *TUBB2A* gene expression and lower paclitaxel sensitivity in cell line models provides biologic evidence that supports this association.

Previous studies suggest that genetic variation could contribute to paclitaxel neurotoxicity risk. In this respect, paclitaxel cytotoxicity heritability is higher than 0.50 and among the highest from a range of the cytotoxic drugs tested in lymphoblastoid cell lines (31). Among the genes that have previously been associated with paclitaxel neurotoxicity risk, most are involved in paclitaxel clearance pathway,

CYP2C8, *CYP3A5*, and *ABCB1* (14, 15, 32). Genes involved in other pathways have also been suggested to influence paclitaxel neurotoxicity. In this respect, 2 haplotypes of *FANCD2*, a DNA repair gene, were associated with the expression of this gene and increased paclitaxel neurologic toxicity (33), suggesting an altered activity to repair chemotherapy-induced DNA damage. However, the precise mechanism by which this enzyme interferes with paclitaxel-induced neuropathy remains to be elucidated, as paclitaxel does not produce DNA breaks, but a potential role for DNA damage following mitotic arrest has been proposed for this drug.

This study constitutes the first evidence supporting that polymorphisms in the therapeutic target of paclitaxel, β-tubulin, can influence the clinical outcome of patients treated with this drug. Changes in β-tubulin isotype composition have been associated with paclitaxel tumor response (20–22). Specifically, increased tumor expression of β-tubulin II has been strongly associated with poor outcome in patients with head and neck carcinoma treated with an induction chemotherapy that contains docetaxel, a paclitaxel analogue (34). Furthermore, *TUBB2A* increased expression has been correlated with decreased drug sensitivity in paclitaxel-resistant cell lines (35). These evidences are in agreement with our study, where we find a very significant correlation between high *TUBB2A* gene expression and lower paclitaxel-induced apoptosis in lymphoblastoid cell lines ($P = 0.001$; Fig. 4). However, it is important to note that the variation in additional genes likely accounts for additional interindividual variability in caspase-3/7 activity ($r^2 = 0.131$; Fig. 4). In a similar way to the cell lines, we found that patients carrying *TUBB2A* polymorphisms leading to increased transcription rate had a decreased risk of developing paclitaxel neurotoxicity (HR, 0.62; 95% CI, 0.42–0.93; $P = 0.021$; Fig. 3). All these data suggest that high amounts of β-tubulin II confer resistance to the action of taxanes. In this regard, the complex expression patterns of the multiple β-tubulin isotypes together with *in vitro* experiments suggest a different functionality and drug sensitivity of the different isotypes (36–38), which could explain higher paclitaxel resistance with increased *TUBB2A* expression.

The great interindividual variability that we found in *TUBB2A* expression reflects the high genetic variability that we found in *TUBB2A* promoter region (Supplementary Table S2). Luciferase activity assays showed that −101C/−112G variants were functional and influenced transcription rate. The close proximity of −101/−112 polymorphisms to the TATA box in *TUBB2A* core promoter together with *in silico* predictions suggesting that several transcription factor–binding sites could be affected by these polymorphisms (Supplementary Fig. S2) further supports the functionality of these variants. Although *TUBB2A* −157G polymorphism was associated with increased *TUBB2A* mRNA content in lymphocytes, it did not affect luciferase activity and we did not find an association between this single-nucleotide polymorphism and the patients' neurotoxicity risk, suggesting that this variant does not influence

paclitaxel effects. However, the allele frequency of this polymorphism is relatively low (0.047) reducing the statistical power, and this variant may just be a marker in linkage disequilibrium with a regulatory variant located in another region of *TUBB2A* promoter. In addition, we cannot rule out that other *TUBB2A* promoter polymorphisms could also be contributing to the observed variability in expression and paclitaxel toxicity risk. Similarly, polymorphisms leading to a variable expression of other neuronal β-tubulins could also influence paclitaxel neurotoxicity. In this respect, we have ruled out variability in β-tubulin I and IVb expression; however, because IVa, IIb, and III are mainly neuron-specific, we could not include them in our study.

In conclusion, in this study, we found a large interindividual variability in *TUBB2A* expression related to the higher transcriptional rate of the variant −101C/−112G *TUBB2A* promoter. Furthermore, cell line models showed that increased *TUBB2A* expression correlated with resistance to paclitaxel, and in patients, we found that −101C/−112G *TUBB2A* regulatory polymorphisms conferred a significantly lower paclitaxel-induced neuropathy risk. This is the first study showing an association between paclitaxel toxicity and regulatory polymorphisms in a therapeutic target of this drug (β-tubulin IIa). If confirmed in independent series, these polymorphisms could be used as markers of paclitaxel-induced peripheral neurotoxicity risk, providing the basis for an individualized paclitaxel pharmacotherapy.

References

1. Rowinsky EK. The development and clinical utility of the taxane class of antimicrotubule chemotherapy agents. Annu Rev Med 1997;48: 353–74.
2. Yvon AM, Wadsworth P, Jordan MA. Taxol suppresses dynamics of individual microtubules in living human tumor cells. Mol Biol Cell 1999;10:947–59.
3. Schiff PB, Fant J, Horwitz SB. Promotion of microtubule assembly *in vitro* by taxol. Nature 1979;277:665–7.
4. Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT. Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. J Am Chem Soc 1971;93:2325–7.
5. Jordan MA, Wilson L. Microtubules as a target for anticancer drugs. Nat Rev Cancer 2004;4:253–65.
6. Risinger AL, Giles FJ, Mooberry SL. Microtubule dynamics as a target in oncology. Cancer Treat Rev 2009;35:255–61.
7. Argyriou AA, Koltzenburg M, Polychronopoulos P, Papapetropoulos S, Kalofonos HP. Peripheral nerve damage associated with administration of taxanes in patients with cancer. Crit Rev Oncol Hematol 2008;66:218–28.
8. Kuroi K, Shimozuma K. Neurotoxicity of taxanes: symptoms and quality of life assessment. Breast Cancer 2004;11:92–9.
9. Nakata T, Yorifuji H. Morphological evidence of the inhibitory effect of taxol on the fast axonal transport. Neurosci Res 1999;35:113–22.
10. Shemesh OA, Spira ME. Paclitaxel induces axonal microtubules polar reconfiguration and impaired organelle transport: implications for the pathogenesis of paclitaxel-induced polyneuropathy. Acta Neuropathol 2010;119:235–48.
11. Theiss C, Meller K. Taxol impairs anterograde axonal transport of microinjected horseradish peroxidase in dorsal root ganglia neurons *in vitro*. Cell Tissue Res 2000;299:213–24.

12. Mielke S, Sparreboom A, Steinberg SM, Gelderblom H, Unger C, Behringer D, et al. Association of Paclitaxel pharmacokinetics with the development of peripheral neuropathy in patients with advanced cancer. Clin Cancer Res 2005;11:4843–50.
13. Rowinsky EK, Eisenhauer EA, Chaudhry V, Arbuck SG, Donehower RC. Clinical toxicities encountered with paclitaxel (Taxol). Semin Oncol 1993;20:1–15.
14. Green H, Soderkvist P, Rosenberg P, Mirghani RA, Rymark P, Lundqvist EA, et al. Pharmacogenetic studies of Paclitaxel in the treatment of ovarian cancer. Basic Clin Pharmacol Toxicol 2009; 104:130–7.
15. Leskela S, Jara C, Leandro-Garcia LJ, Martinez A, Garcia-Donas J, Hernando S, et al. Polymorphisms in cytochromes P450 2C8 and 3A5 are associated with paclitaxel neurotoxicity. Pharmacogenomics J 2011;11:121–9.
16. Leandro-Garcia LJ, Leskela S, Landa I, Montero-Conde C, Lopez-Jimenez E, Leton R, et al. Tumoral and tissue-specific expression of the major human beta-tubulin isotypes. Cytoskeleton (Hoboken) 2010;67: 214–23.
17. Freson K, De Vos R, Wittevrongel C, Thys C, Defoor J, Vanhees L, et al. The TUBB1 Q43P functional polymorphism reduces the risk of cardiovascular disease in men by modulating platelet function and structure. Blood 2005;106:2356–62.
18. Jaglin XH, Poirier K, Saillour Y, Buhler E, Tian G, Bahi-Buisson N, et al. Mutations in the beta-tubulin gene TUBB2B result in asymmetrical polymicrogyria. Nat Genet 2009;41:746–52.
19. Tischfield MA, Baris HN, Wu C, Rudolph G, Van Maldergem L, He W, et al. Human TUBB3 mutations perturb microtubule dynamics, kinesin interactions, and axon guidance. Cell 2010;140:74–87.
20. Ferrandina G, Zannoni GF, Martinelli E, Paglia A, Gallotta V, Mozzetti S, et al. Class III beta-tubulin overexpression is a marker of poor clinical

outcome in advanced ovarian cancer patients. Clin Cancer Res 2006; 12:2774–9.

21. Seve P, Dumontet C. Is class III beta-tubulin a predictive factor in patients receiving tubulin-binding agents? Lancet Oncol 2008;9: 168–75.

22. Seve P, Mackey J, Isaac S, Tredan O, Souquet PJ, Perol M, et al. Class III beta-tubulin expression in tumor cells predicts response and outcome in patients with non-small cell lung cancer receiving paclitaxel. Mol Cancer Ther 2005;4:2001–7.

23. McCoy JP Jr. Handling, storage, and preparation of human blood cells. Curr Protoc Cytom 2001;Chapter 5:Unit 5.1.

24. Green H, Khan MS, Jakobsen-Falk I, Avall-Lundqvist E, Peterson C. Impact of CYP3A5*3 and CYP2C8-HapC on paclitaxel/carboplatin-induced myelosuppression in patients with ovarian cancer. J Pharm Sci 2011;100:4205–9.

25. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402–8.

26. Njiaju UO, Gamazon ER, Gorsic LK, Delaney SM, Wheeler HE, Im HK, et al. Whole genome studies identify solute carrier transporters in cellular susceptibility to paclitaxel. Pharmacogenet Genomics 2012;22:498–507.

27. Zhang W, Duan S, Kistner EO, Bleibel WK, Huang RS, Clark TA, et al. Evaluation of genetic variation contributing to differences in gene expression between populations. Am J Hum Genet 2008;82:631–40.

28. Huang RS, Duan S, Bleibel WK, Kistner EO, Zhang W, Clark TA, et al. A genome-wide approach to identify genetic variants that contribute to etoposide-induced cytotoxicity. Proc Natl Acad Sci U S A 2007;104: 9758–63.

29. Seidman AD, Berry D, Cirrincione C, Harris L, Muss H, Marcom PK, et al. Randomized phase III trial of weekly compared with every-3-weeks paclitaxel for metastatic breast cancer, with trastuzumab for all HER-2 overexpressors and random assignment to trastuzumab or not in HER-2 nonoverexpressors: final results of Cancer and Leukemia Group B protocol 9840. J Clin Oncol 2008;26:1642–9.

30. Wen Y, Gorsic LK, Wheeler HE, Ziliak DM, Stephanie Huang R, Eileen Dolan M. Chemotherapeutic-induced apoptosis: a phenotype for pharmacogenomics studies. Pharmacogenet Genomics 2011;21: 476–88.

31. Peters EJ, Motsinger-Reif A, Havener TM, Everitt L, Hardison NE, Watson VG, et al. Pharmacogenomic characterization of US FDA-approved cytotoxic drugs. Pharmacogenomics 2011;12: 1407–15.

32. Sissung TM, Mross K, Steinberg SM, Behringer D, Figg WD, Sparreboom A, et al. Association of ABCB1 genotypes with paclitaxel-mediated peripheral neuropathy and neutropenia. Eur J Cancer 2006; 42:2893–6.

33. Sucheston LE, Zhao H, Yao S, Zirpoli G, Liu S, Barlow WE, et al. Genetic predictors of taxane-induced neurotoxicity in a SWOG phase III intergroup adjuvant breast cancer treatment trial (S0221). Breast Cancer Res Treat 2011;130:993–1002.

34. Cullen KJ, Schumaker L, Nikitakis N, Goloubeva O, Tan M, Sarlis NJ, et al. beta-Tubulin-II expression strongly predicts outcome in patients receiving induction chemotherapy for locally advanced squamous carcinoma of the head and neck: a companion analysis of the TAX 324 trial. J Clin Oncol 2009;27:6222–8.

35. Tegze B, Szallasi Z, Haltrich I, Penzvalto Z, Toth Z, Liko I, et al. Parallel evolution under chemotherapy pressure in 29 breast cancer cell lines results in dissimilar mechanisms of resistance. PLoS One 2012;7: e30804.

36. Banerjee A, Roach MC, Trcka P, Luduena RF. Increased microtubule assembly in bovine brain tubulin lacking the type III isotype of beta-tubulin. J Biol Chem 1990;265:1794–9.

37. 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.

38. Panda D, Miller HP, Banerjee A, Luduena RF, Wilson L. Microtubule dynamics in vitro are regulated by the tubulin isotype composition. Proc Natl Acad Sci U S A 1994;91:11358–62.

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