Predictive Biomarkers and Personalized Medicine

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.
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, isoform 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 ΔΔCT 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...
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 Ila gene (−389 to −15, nucleotide positions referring to TUBB2A translation start site ATG, +1) using specific primers that introduced XhoI and HindIII 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 UIT (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
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 U test was used to compare the log$_2$ expression of these polymorphisms in Caucasian population are 0.28, 0.72, and 0.001, respectively (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>G, −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).

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>G, −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).

**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>G, −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).

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 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 −101C/−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 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/m² weekly scheme being more neurotoxic than 150 to 175 mg/m² 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
Methods. The graph shows a linear model comparing log2 expression in the same cell lines, as described in Materials and Methods. The graph shows a linear model comparing log2 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 FANC D2, 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 (r² = 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L.J. Leandro-Garcia, C. Jara, H. Green, E. Avall-Lundqvist, H.E. Wheeler, M.E. Dolan, A. Cascon, M. Robledo, C. Rodriguez-Antonia
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