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Tesis doctoral

Identificación de marcadores predictivos de toxicidad y eficacia del paclitaxel: regulación y variación genética de la β-tubulina y estudio de asociación del genoma completo

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Tesis doctoral que presenta para optar al título de
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La presente Tesis Doctoral se realizó en el Centro Nacional de Investigaciones Oncológicas (CNIO) de Madrid entre los años 2007 y 2012 bajo la supervisión de la Dra. Cristina Rodríguez González de Antona y la Dra. Mercedes Robledo Batanero

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RESUMEN

El paclitaxel es un agente de unión a microtúbulos ampliamente usado en la clínica para el tratamiento de tumores sólidos. Existen grandes diferencias interindividuales tanto en la eficacia como en los efectos adversos de este fármaco que, en parte, podrían ser explicadas por la variabilidad del genoma humano. Hasta la actualidad, los estudios farmacogenéticos del paclitaxel han utilizado aproximaciones de genes candidatos, centrándose fundamentalmente en su ruta farmacocinética. En esta tesis hemos adoptado dos tipos de aproximaciones para la identificación de posibles marcadores asociados a la toxicidad y eficacia del paclitaxel: en primer lugar, hemos estudiado la regulación y variación genética de los isotipos de β-tubulina, la diana terapéutica de éste fármaco; en segundo lugar, hemos llevado a cabo un estudio de asociación del genoma completo.

Con respecto a la regulación y la variación genética de los isotipos de β-tubulina, esta tesis aporta información clave sobre la expresión de los isotipos más relevantes de β-tubulina en diversos tejidos humanos normales y tumorales. Esta información podría ayudar al diseño de nuevos fármacos de unión a microtúbulos específicos de isotipo, de mayor eficacia y menor toxicidad. Asimismo, hemos descubierto que una baja expresión de los miembros de la familia miR-200 se asocia a una alta expresión de la β-tubulina III y a una mala respuesta y pronóstico de los tumores de ovario tratados con paclitaxel/ carboplatino. Hemos encontrado una importante variabilidad interindividual en la expresión de la β-tubulina II, causada por los polimorfismos -101C>T/-112G>A en el promotor del gen y demostramos que estos polimorfismos protegen frente al desarrollo de la neuropatía inducida por paclitaxel. Finalmente, hemos demostrado que la β-tubulina VI es un isotipo específico hematológico sujeto a una importante variabilidad genética. También descubrimos que el polimorfismo T274M de la β-tubulina VI disminuye el efecto del paclitaxel en los microtúbulos y aportamos evidencias que sugieren que este polimorfismo podría constituir un marcador de mielotoxicidad en pacientes tratados con paclitaxel.

Con respecto al estudio de asociación del genoma completo, éste se centró en la neuropatía causada por el paclitaxel. En él confirmamos que el polimorfismo rs7349683 del gen *EPHA5* es un marcador de la neuropatía inducida por paclitaxel ($P=1.4x10^{-9}$). También proponemos que variantes comunes en otros genes *EPHA* y en el locus *LIMK2* podrían desempeñar un papel importante para esta toxicidad. Todo ello sugiere que genes involucrados en la función y reparación de los nervios periféricos, no estudiados previamente en aproximaciones de genes candidatos, podrían contribuir de forma importante en la susceptibilidad genética a desarrollar esta toxicidad.

En resumen, los marcadores identificados en esta tesis doctoral podrían tener una importante relevancia clínica, contribuyendo a la individualización de la farmacoterapia con paclitaxel mediante la clasificación de los pacientes de acuerdo a su riesgo de desarrollar toxicidad o falta de eficacia durante el tratamiento.

SUMMARY

Paclitaxel is a microtubule-binding agent widely used in the clinic for the treatment of solid tumors. There are large inter-individual differences in the efficacy and adverse effects of this drug that could partly be explained by the variability in the human genome. So far, paclitaxel pharmacogenetic studies have used candidate gene strategies, mainly focusing on its pharmacokinetic pathway. In this thesis we have adopted two approaches for the identification of markers associated with the toxicity and efficacy of paclitaxel: first, we have studied the regulation and genetic variation of the β-tubulin isotypes, the therapeutic target of this drug; second, we have carried out a whole genome association study.

With regards to the regulation and genetic variation of the β-tubulin isotypes, this thesis provides novel information about the expression of the most relevant β-tubulin isotypes in human normal and tumoral tissues. This information may help to design new isotype-specific microtubule-binding drugs with increased efficacy and less toxicity. We also discovered that a low expression of the miR-200 family members is associated with increased expression of β-tubulin III, and with poor response and prognosis of ovarian tumors treated with paclitaxel/ carboplatin. We have found an important inter-individual variability in the expression of β-tubulin II, caused by the polymorphisms -101C>T/-112G>A in the promoter region of the gene, and we have shown that these polymorphisms protect against paclitaxel-induced neuropathy. Finally, we have demonstrated that β-tubulin VI is a hematological isotype with a significant genetic variability. We also discovered that β-tubulin VI T274M polymorphism diminished the effect of paclitaxel on the microtubules, and we provide evidences that suggest that this polymorphism could be a marker of myelotoxicity in patients treated with paclitaxel.

Regarding the whole genome association study, this was focused on the neuropathy caused by paclitaxel. In this study we confirm that *EPHA5* rs7349683 is a marker of paclitaxel-induced neuropathy ($P= 1.4x10^{-9}$). We also propose that common variants in other *EPHA* genes and *LIMK2* locus could play an important role for this toxicity. Altogether, this data suggests that genes involved in the function and repair of peripheral nerves, not previously studied in candidate gene approaches, could contribute significantly to the genetic susceptibility to develop this toxicity.

In summary, the markers identified in this PhD thesis could have an important clinical relevance, contributing to the individualization of paclitaxel pharmacotherapy by stratifying patients according to their risk of toxicity and lack of efficacy during treatment.

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ABREVIATURAS

ABC: Cassette de unión de ATP (del inglés *ATP Binding Cassette*)

ADL: Actividades de la vida diaria (del inglés *Activities of Daily Living*)

ADN / DNA: Ácido Desoxirribonucleico (en inglés *Deoxyribonucleic Acid*)

ADNc / cDNA: ADN copia (en inglés *copy DNA*)

ADRs: Reacciones Adversas (del inglés *Adverse Drug Reactions*)

ARN / RNA: Ácido Ribonucleico (en inglés *Ribonucleic Acid*)

ARNm / mRNA: ARN Mensajero (en inglés *Messenger RNA*)

ATP: Adenosin trifosfato (del inglés *Adenosine Triphosphate*)

AUC: Area Bajo la Curva (del inglés *Area Under Curve*)

CNVs: Variaciones en el Número de Copias o polimorfismos de ganancia o pérdida de regiones mayores de 1 Kb (del inglés *Copy Number Variants*)

CR: Respuesta Completa (del inglés *Complete Response*)

IC95% / 95%CI: Intervalo de Confianza al 95% (del inglés *Confidence Interval*)

CTCAE: Criterios de Terminología Común para los Eventos Adversos (del inglés *Common Terminology Criteria for Adverse Events*)

EMEA: Agencia Europea del Medicamente (del inglés *European Medicines Agency*)

EMT: Transición de Epitelio a Mesénquima (del inglés *Epithelial to Mesenchymal Transition*)

FDA: Agencia de Control de Alimentos y Medicamentos en Estados Unidos o (del inglés *Food and Drug Administration*)

GCSFs: Factores Estimulantes de Colonias de Granulocitos (del inglés *Granulocyte Colony Stimulating Factors*)

GWAS: Estudios de Asociación del Genoma Completo (del inglés *Genome Wide Association Studies*)

HIF: Factor Inducido por Hipoxia (del inglés *Hipoxia Inducible Factor*)

HR: Proporción de Riesgo (del inglés *Harzard Ratio*)

In-dels: Polimorfismos de pequeñas Inserciones y Deleciones menores de 1Kb

LLN: Límite inferior normal (del inglés *Lower Limit Normal*)

MAPS: Proteínas Asociadas a Microtúbulos (del inglés *Microtubule-Associated ProteinS*)

miARN / miRNA: Micro ARN (del inglés *MicroRNA*)

MRP: Proteínas de resistencia a Múltiples Fármacos (del inglés *Multidrug Resistant Proteins*)

ncRNAs: ARNs No Codificantes (del inglés *Non Coding RNAs*)

NGS: Secuenciación de Nueva Generación (del inglés *Next Generation Sequecing*)

OS: Supervivencia Global (del inglés *Overall Survival*)

PCR: Reacción en Cadena de la Polimerasa (del inglés *Polymerase Chain Reaction*)

PD: Farmacodinámica (del inglés *Pharmacodynamics*)

PFS: Supervivencia Libre de Progresión (del inglés *Progression Free Survival*)

PK: Farmacocinética (del inglés *Pharmacokinetics*)

qRT-PCR: PCR Cuantitativa a Tiempo Real (del inglés *quantitative Real Time - Polymerase Chain Reaction*)

SNP: Polimorfismo de un Solo Nucleótido (del inglés *Single Nucleotide Polymorphism*)

STRs: Microsatélites o Repeticiones Cortas en Tándem (del inglés *Short Tandem Repeats*)

tagSNPs: SNPs Marcadores (del inglés *Tag Single Nucleotide Polymorphisms*)

UTR: Región No Traducida (del inglés *Un-Translated Region*)

VNTRs: Repeticiones en Tándem de Número Variable (del inglés *Variable Number Tandem Repeats*)

INTRODUCCIÓN

INTRODUCCIÓN

PACLITAXEL Y β-TUBULINAS

I.1. Agentes de unión a tubulina

Un amplio número de moléculas con estructuras muy diversas, en algunos casos obtenidas de fuentes naturales, tienen la capacidad de unirse a la tubulina, el componente fundamental de los microtúbulos celulares, alterando su polimerización y afectando su dinámica. Dado que en las células en división los microtúbulos forman el huso mitótico, la unión de estas moléculas a los microtúbulos provoca el arresto del ciclo celular en mitosis y finalmente causa la muerte celular (Noble 1958, Schiff 1979, Wani 1971). Esta característica explica en parte que se hayan desarrollado diversos agentes de unión a tubulina para tratar el cáncer, con un uso amplio y eficiente. Además, el efecto antineoplásico de estos fármacos se ve reforzado con un efecto anti-angiogénico inhibidor de la vasculatura tumoral, aunque las bases moleculares de este último se desconocen (Lippert 2007, Zhou 2005).

Entre estos agentes antineoplásicos de unión a microtúbulos se cuentan los taxanos. Estas moléculas, inicialmente aisladas hace cuarenta años (Wani 1971), son usadas frecuentemente, en monoterapia o en combinación con otros fármacos, como primera línea en el tratamiento del cáncer de ovario, mama, pulmón y próstata. La unión de los taxanos en el lumen interior de los microtúbulos, en la subunidad β-tubulina del heterodímero αβ-tubulina, promueve la estabilización de éstos, impidiendo la división celular y conduciendo a apoptosis (Schiff 1979, Yvon 1999). Los dos tipos de taxanos más comúnmente usados son el paclitaxel (Taxol®, Bristol-Meyers Squibb), inicialmente aislado de la corteza del tejo del Pacífico (*Taxus brevifolia*) (Rowinsky 1997), y su análogo semi-sintético docetaxel (Taxotere®, Sanofi-Aventis), presente en las acículas del tejo europeo (*Taxus baccata*) (Diaz 1993, Saloustros 2008) (Figura 1). También se han desarrollado nuevas formulaciones de taxanos de administración oral, como el nab-paclitaxel. Entre otros taxanos cuyo uso es menos común se incluye el cabazitaxel (Jevtana®, Sanofi-Aventis). Actualmente existen nuevas moléculas en ensayos clínicos o en fase preclínica como milataxel (Lockhart 2007, Ramanathan 2008), larotaxel (Dieras 2008, Yamamoto 2009), ortataxel (Minderman 2004), tesetaxel (Baas 2008, Shionoya 2003) o genexol-PM (Kim 2004, Park 2004), entre otros.

I.2. Paclitaxel

Actualmente el paclitaxel (Taxol®, Bristol-Meyers Squibb) está aprobado por la Agencia de Control de Alimentos y Medicamentos en Estados Unidos o FDA (del inglés *Food and Drug Administration*) y la Agencia Europea del Medicamente o EMEA (del inglés *European Medicines Agency*) para el tratamiento del cáncer de ovario, mama y pulmón. La vía de administración del Taxol es intravenosa, ya que debido a su pobre solubilidad acuosa es necesario suministrarlo con excipientes como el aceite de ricino

polietoxilado Cremophor (Cremophor EL) y etanol. El Cremophor EL es responsable de las reacciones de hipersensibilidad que se observan con este compuesto (Gelderblom 2001).

El paclitaxel unido a albúmina, o nab-paclitaxel (Abraxane®, Abraxis BioScience) está incluido en diversos ensayos de fase III. El nab-paclitaxel ha obtenido mejor respuesta y tolerancia que el docetaxel en pacientes con cáncer de mama metastásico tratados en primera línea (Gradishar 2009). El desarrollo de nuevos fármacos como el nab-paclitaxel responde a la necesidad de encontrar nuevas formulaciones que reduzcan los problemas asociados a la baja solubilidad y tolerancia de los taxanos clásicos.

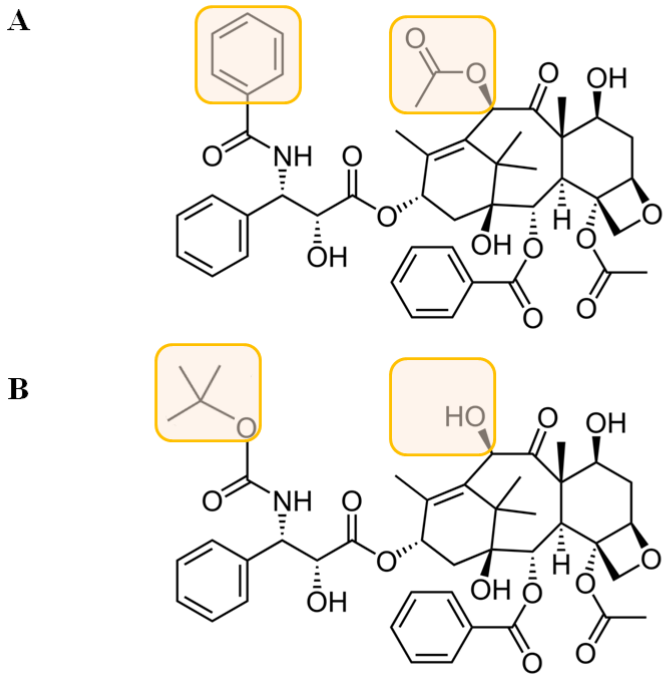

Figura 1. Estructura molecular de los taxanos. (A) paclitaxel y (B) docetaxel. Los residuos que diferencian a ambas moléculas están enmarcados en naranja.

I.3. Problemas clínicos asociados al uso del paclitaxel

Aunque el paclitaxel se lleva utilizando desde los años 70 como un eficaz tratamiento para diversos tipos de cánceres, presenta serias limitaciones clínicas debidas tanto a las toxicidades asociadas a su uso, como a la resistencia innata o adquirida al fármaco.

I.3.a. Toxicidades

La **neuropatía periférica** es la toxicidad limitante de dosis más común del paclitaxel (Jordan 2004, Risinger 2009). Entre un 60 y un 80% de los pacientes tratados con este fármaco desarrollan neuropatía periférica (Balayssac 2011, Gutierrez-Gutierrez 2010, Ocean 2004, Quasthoff 2002). El tipo y

gravedad de esta toxicidad neurológica se clasifica dependiendo de los efectos observados en el individuo y la afectación de sus actividades diarias (Tabla 1). La neurotoxicidad inducida por paclitaxel es predominantemente sensorial y se desarrolla como una neuropatía axonal distal, simétrica, debilitante y dolorosa (Argyriou 2008, Kuroi 2004). Aunque hasta el momento los mecanismos causales de esta toxicidad no han sido determinados con precisión, es claro que el transporte axonal mediado por microtúbulos se ve afectado (Nakata 1999, Shemesh 2010, Theiss 2000). La neurotoxicidad del paclitaxel es dosis acumulativa y existen diversos factores clínicos que aumentan el riesgo a desarrollarla: regímenes semanales, existencia de *diabetes mellitus*, tratamientos previos con agentes neurotóxicos, fármacos anti-retrovirales, enfermedad hepática crónica, alcoholismo, hipotiroidismo, déficits nutricionales y polineuropatías previas hereditarias o adquiridas (Argyriou 2008, Chaudhry 2003, Mielke 2005, Rowinsky 1993). Existe una gran variabilidad interindividual en la neurotoxicidad inducida por paclitaxel, de modo que entre los pacientes que reciben paclitaxel en protocolos y cantidades similares algunos pacientes son asintomáticos mientras que otros sufren neuropatías graves que conducen a retrasos o suspensiones del tratamiento. Los síntomas normalmente desaparecen meses después de finalizar el tratamiento con paclitaxel, pero en los casos más severos el daño en los nervios periféricos es irreversible. Nuestro grupo y otros han investigado la contribución de variantes genéticas en la ruta farmacocinética del paclitaxel al riesgo a desarrollar esta toxicidad (Green 2009, Leskela 2011, Sissung 2006), pero todavía una gran parte de la variabilidad inter-individual de la neurotoxicidad inducida por paclitaxel se debe a causas desconocidas y es impredecible.

La **mielotoxicidad** es un efecto adverso común a muchos agentes citotóxicos que basan su acción en la mayor tasa de proliferación de las células tumorales, ya que en la médula ósea existe una renovación celular constante. El tipo y gravedad de la toxicidad hematológica se clasifica dependiendo del tipo celular afectado y del recuento final de células en la sangre (Tabla 1). La neutropenia es el evento más frecuente y severo asociado a regímenes que incluyen taxanos. El paclitaxel en monoterapia (175 mg/m^2, cada 21 días) puede resultar en neutropenias, anemias y trombopenias grado 2 o mayor en un 20, 10 y 1% de los pacientes, respectivamente (Albain 2008, Di Leo 2008). La incidencia de la mielotoxicidad se ha visto significativamente reducida en los últimos años por el uso de tiempos de infusión cortos y la coadministración de factores estimulantes de colonias de granulocitos o GCSFs (del inglés *Granulocyte Colony Stimulating Factors*) (Eisenhauer 1994, Rowinsky 1993). Sin embargo, en sus formas más severas, la mielotoxicidad sigue constituyendo un factor limitante de dosis, que puede conducir a suspensiones del tratamiento comprometiendo el éxito de la terapia.

I.3.b. Eficacia terapéutica

Las resistencias a fármacos, innatas y adquiridas, se han convertido en uno de los principales problemas en la lucha contra el cáncer. En el caso del paclitaxel, se han descrito varios mecanismos de resistencia. La identificación y estudio de estos procesos son de una gran importancia para el desarrollo

de nuevos y más potentes agentes de unión a microtúbulos. Hasta ahora se han descrito los siguientes mecanismos:

1) **Transportadores de membrana dependientes de ATP**. Constituyen el primer mecanismo de resistencia desarrollado por las células tumorales cuando son expuestas a agentes de unión a microtúbulos. La sobreexpresión en las células tumorales de la glicoproteína P es la causa principal de las resistencias a paclitaxel. Esta proteína (codificada por el gen *ABCB1*) bombea los fármacos fuera de las células tumorales, reduciendo sus concentraciones intracelulares y por tanto su actividad citotóxica (Horwitz 1993). Además de la glicoproteína P, el paclitaxel es también sustrato de las proteínas de resistencia a múltiples fármacos 2 y 7 (MRP2 y MRP7, respectivamente, del inglés *Multidrug Resistant Proteins*) (Hopper-Borge 2004, Huisman 2005). Para contrarrestar este efecto, se ha intentado aumentar la eficacia de las terapias combinando el paclitaxel con inhibidores de la glicoproteína P, pero aún no se han alcanzado resultados clínicos significativos.

2) **Deficiencias en la señalización apoptótica**. Se ha descrito que el paclitaxel podría ser más activo en las células que han perdido la función de la proteína supresora de tumores p53 (un evento común en células tumorales) (Wahl 1996) mediante una regulación alterada de la composición y dinámica de los microtúbulos (Galmarini 2003). Sin embargo, existe controversia sobre su efecto en la sensibilidad al paclitaxel (King 2000, Malamou-Mitsi 2006). También se ha descrito que la inhibición farmacológica del regulador apoptótico Bcl-X_L aumenta la sensibilidad al paclitaxel (Shoemaker 2006), confirmando que el proceso de apoptosis está directamente implicado en la sensibilidad a este fármaco.

Tabla 1: Criterios de Terminología Común para los eventos adversos versión 3.0 (CTCAE[a] v3,0).

Toxicidad		Grado 1 (Leve)	Grado 2 (Moderado)	Grado 3 (Severo)	Grado 4 (Riesgo de muerte o incapacitante)	Grado 5 (Muerte)
Hematológica	Hemoglobina (Anemia)	<LLN[b] – 6,2 mmol/L <LLN - 100 g/L	<6,2 - 4,9 mmol/L <100 - 80 g/L	<4,9 - 4,0 mmol/L <80 - 65 g/L	<4,0 mmol/L <65 g/L	Muerte
	Leucocitos (Leucopenia)	<LLN – 3,0 x 10^9 /L	<3,0 – 2,0 x 10^9 /L	<2,0 - 3,0 x 10^9 /L	<1,0 x 10^9 /L	Muerte
	Neutrófilos (Neutropenia)	<LLN – 1,5 x 10^9 /L	<1.5 – 1.0 x 10^9 /L	<1,0 – 0,5 x 10^9 /L	<0,5 x 10^9 /L	Muerte
	Plaquetas (Trombopenia)	<LLN – 75,0 x 10^9 /L	<75,0 – 50,0 x 10^9 /L	<50,0 – 25,0 x 10^9 /L	<25,0 x 10^9 /L	Muerte
Neurológica	Neuropatía motora	Asintomática; debilidad detectada sólo mediante examen	Debilidad sintomática interfiriendo con la función, pero sin interferir con ADL[c]	Debilidad que interfiere con ADL; inmovilización o asistencia para caminar indicadas	Riesgo de muerte; incapacitante (parálisis)	Muerte
	Neuropatía sensorial	Asintomática; pérdida de reflejos del tendón o parestesia (incluyendo hormigueo)	Alteración sensorial o parestesia (incluyendo hormigueo), interfiriendo con la función, pero sin interferir con ADL	Alteración sensorial o parestesia interfiriendo con ADL	Incapacitante	Muerte

[a]CTCAE (del inglés *Common Terminology Criteria for Adverse Events*);
[b]LLN: Límite inferior normal (del inglés *Lower Limit Normal*);
[c]ADL: Actividades de la vida diaria (del inglés *Activities of Daily Living*)

3) **Alteraciones en microtúbulos**. En modelos celulares *in vitro* se ha observado que mutaciones en la β-tubulina I alteran la unión de diversos fármacos de unión a microtúbulos y pueden conferir resistencia a éstos (Giannakakou 2000, Giannakakou 1997, Kavallaris 2001, Verrills 2003). Sin embargo, en tumores no parece que este mecanismo juegue un papel importante (Berrieman 2004). Por otro lado, se han observado cambios en la expresión de algunos isotipos de β-tubulina en células malignas. Se ha sugerido que la función de los distintos isotipos es distinta y hay evidencias que sugieren que cambios en los patrones de expresión de los isotipos de β-tubulina podrían estar involucrados en la resistencia a diversos fármacos. En particular, la sobreexpresión del isotipo III se ha asociado con una peor respuesta clínica a diferentes fármacos de unión a microtúbulos (Ferrandina 2007, Ferrandina 2006, Hasegawa 2003, Mozzetti 2005, Seve 2008, Seve 2005, Seve 2007, Seve 2007).

4) **Otros mecanismos**. Alteraciones en los niveles de expresión de varias proteínas asociadas a distintos procesos celulares en los que la dinámica de los microtúbulos juega un importante papel, tales como transporte intracelular, señalización o ciclo celular, también podrían afectar la sensibilidad al paclitaxel. Constituyen ejemplos de este tipo, variaciones en la expresión de proteínas como prohibitina, glutatión transferasa S π, defensinas α, GTSE1 o HIF1-α (Bauer 2010, Bublik 2010, Huang 2009, Patel 2010, Townsend 2003). También se han descrito diversos microARNs que pueden contribuir a la resistencia al paclitaxel: el miR-125b confiere resistencia a paclitaxel mediante la alteración del nivel de expresión de BAK1 (Zhou 2010), y el mir-148a mediante la de MSK1 (Fujita 2010).

I.4. β-tubulinas

La diana terapéutica del paclitaxel es la β-tubulina. Ésta molécula forma junto con la α-tubulina dímeros que polimerizan en estructuras llamadas microtúbulos. El paclitaxel estabiliza los microtúbulos a través de un sitio de unión en la β-tubulina localizado en el lumen interno de su estructura (Rao 1999) (Figura 2). En mamíferos existen al menos seis isotipos de α- y ocho de β-tubulinas, que están codificados por distintos genes (Lopata 1987, Luduena 1998). Los genes de la familia de las β-tubulinas están constituidos por cuatro exones y tres intrones, a diferencia de sus pseudogenes no funcionales, que carecen de todos o la mayoría de los intrones (Berrieman 2004). Los isotipos de β-tubulina están muy conservados entre especies. Sus secuencias proteicas se diferencian principalmente en los 15-20 aminoácidos del extremo C-terminal, que es la región de unión para una serie de proteínas asociadas a microtúbulos o MAPS (del inglés *Microtubule-Associated ProteinS*) (Figura 3) (Sullivan 1986).

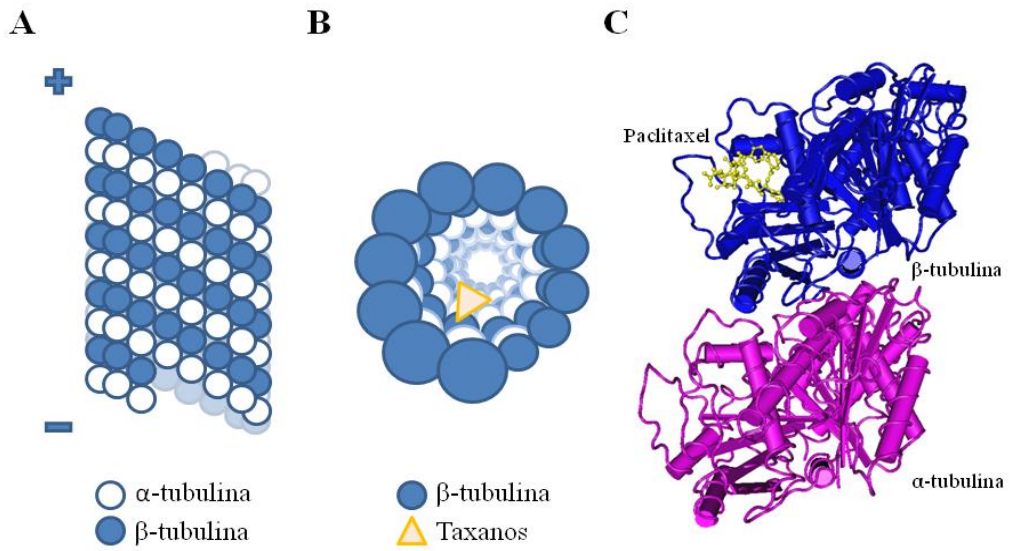

Figura 2. Microtúbulos y sitio de unión del paclitaxel. A) Los microtúbulos están compuestos de heterodímeros de α-β-tubulina. B) Los taxanos se unen a la subunidad β-tubulina en la superficie interna del microtúbulo. C) Sitio de unión del paclitaxel en la subunidad β-tubulina.

I.4.a. Expresión de los isotipos humanos de β-tubulina

El patrón de expresión de los diferentes isotipos de β-tubulina ha sido estudiado en tejidos de distintos mamíferos (principalmente pollo, rata y ratón) y líneas celulares de origen humano (Cleveland 1987, Hiser 2006), aunque de manera incompleta. Ello se debe principalmente al uso de anticuerpos que no son capaces de reconocer de forma específica y única a los distintos isotipos. No obstante, estos estudios han demostrado que la expresión de algunos isotipos de β-tubulina parece ser constitutiva: clases I, IVb y quizá V; mientras que otros isotipos parecen tener una expresión restringida a algunos tejidos específicos: clases II, III y IVa a tejido neuronal (Cleveland 1987, Lopata 1987, Sullivan 1986, Verdier-Pinard 2005) y clase VI a tejido hematopoyético (Wang 1986). En cualquier caso, hasta la fecha no se ha realizado una caracterización integral de la expresión de los isotipos de β-tubulina en tejidos humanos.

A

Gen	*TUBB* (HM40)	*TUBB1* (Hβ1)	*TUBB2A*	*TUBB2B* (Hβ9)	*TUBB2C* (Hβ2)	*TUBB3* (Hβ4)	*TUBB4* (Hβ5)	*TUBB6*
Proteína	β-tubulina I	β-tubulina VI	β-tubulina IIa	β-tubulina IIb	β-tubulina IVb	β-tubulina III	β-tubulina IVa	β-tubulina V

B

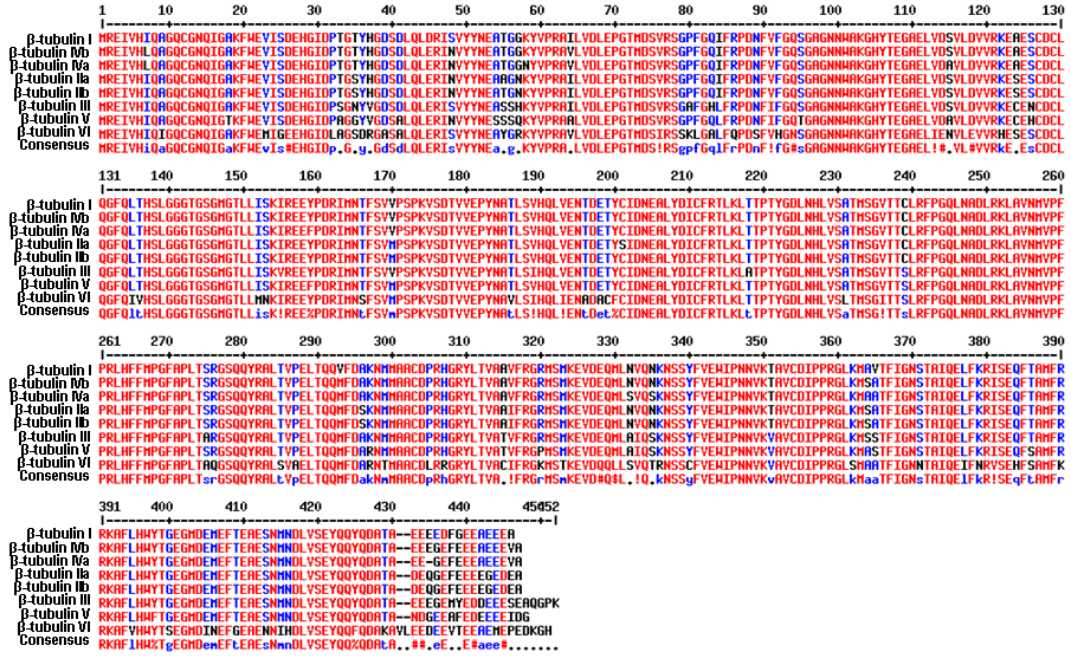

Figura 3. **Isotipos humanos de β-tubulina.** A) Nomenclatura y correspondencia entre gen y proteína. B) Alineamiento de las secuencias de las proteínas de los 8 isotipos de β-tubulina.

I.4.b. Regulación de la expresión de los isotipos de β-tubulina

La regulación de la expresión de los diferentes isotipos de β-tubulina es todavía poco conocida. En particular, los mecanismos moleculares que conducen a la sobreexpresión del isotipo III en tumores se han convertido en sujeto de varias investigaciones. Parece ser que el factor inducido por hipoxia 1 o HIF-1 (del inglés *Hipoxia Inducible Factor 1*) podría estar implicado en la regulación de la expresión de la β-tubulina III en carcinoma de ovario (Raspaglio 2008). Otros factores que podrían ser importantes son las modificaciones epigenéticas en tumores de ovario (Izutsu 2008) o en melanoma (Akasaka 2009). Estudios recientes han puesto de manifiesto la importante contribución de los microARNs a la regulación de la expresión génica. Específicamente, se ha demostrado que el miR-200c tiene un sitio de unión en la región no traducida 3' o 3'UTR (del inglés *Un-Translated Region*) del mensajero de la β-tubulina III y que la sobreexpresión de este microARN en líneas celulares disminuye la cantidad de proteína de β-tubulina III (Cochrane 2010, Cochrane 2009). Merece la pena destacar que la familia de microARNs 200, compuesta por 5 microARNs (miR-141, miR-200a, miR-200b, miR-200c y miR-429) y localizada en dos *clusters* en el genoma humano, está involucrada en la transición de epitelio a mesénquima o EMT (del inglés *Epithelial to Mesenchymal Transition*) mediante la regulación de la expresión de la cadherina E a través de la supresión de los factores de transcripción ZEB1 y ZEB2 (Gregory 2008, Korpal 2008, Park

2008). Un estudio reciente con tumores de ovario avanzados describió que una alta expresión de los microARNs 200a, 200b y 429 se asociaba con una mejor supervivencia (Hu 2009). No obstante, otro estudio que incluía carcinomas serosos de ovario encontró que una alta expresión de los miembros de la familia de microARNs 200 se asociaba con peor pronóstico (Nam 2008). Por lo tanto, aunque hay evidencias que sugieren que la familia de microARNs 200 podría estar jugando un papel en el pronóstico tumoral y en la respuesta a los fármacos de unión a microtúbulos mediante la regulación de los isotipos de β-tubulina, la relevancia de estos microARNs como marcadores clínicos tiene todavía que ser esclarecida.

I.4.c. Variabilidad genética en los isotipos de β-tubulina

Los isotipos de β-tubulina, aunque están altamente conservados y tienen secuencias muy similares, difieren en la región C-terminal de la proteína que parece conferir distinta estabilidad y capacidad de polimerización a los microtúbulos. El hecho de que mutaciones pocos comunes en los isotipos de β-tubulina IIb y III sean causantes de un amplio espectro de desórdenes neuronales severos (Jaglin 2009, Tischfield 2010), sugiere que cada isotipo tiene funciones específicas que no pueden ser compensadas por los otros. También se sabe que el cerebro es el tejido humano que contiene las mayores cantidades de β-tubulina, probablemente reflejando la importancia del citoesqueleto para las diversas funciones que las neuronas desarrollan. Por otra parte, estudios en ratones modificados genéticamente que no expresan la proteína β-tubulina VI (ratones *knockout* para el gen *TUBB1*) han demostrado un papel crucial de este isotipo en la síntesis, estructura y función de las plaquetas (Italiano 2003, Schwer 2001). Existe también un estudio que describe un paciente con una variante rara de cambio de aminoácido (p.R318W) en la β-tubulina VI que da lugar a una macrotrombocitopenia congénita (Kunishima 2009).

Debido al papel crucial que juegan las β-tubulinas en la célula, éstas están altamente conservadas y no se han descrito variantes genéticas comunes en la población que den lugar a cambios en la proteína. Sin embargo, el isotipo de β-tubulina VI es una excepción. En este isotipo se ha descrito un polimorfismo de cambio de aminoácido (p.Q43P), que se asocia con el riesgo de enfermedades cardiovasculares debido a una estructura y función plaquetaria alterada (Freson 2005, Navarro-Nunez 2007).

En líneas celulares se han detectado mutaciones en los isotipos de β-tubulina que confieren resistencia a agentes de unión a microtúbulos (Giannakakou 1997, Kavallaris 2001, Verrills 2003). Esto junto con el hecho de que variaciones en la composición tumoral de las β-tubulinas se han asociado a una resistencia a estos fármacos (Ferrandina 2006, Rosell 2003, Seve 2008, Seve 2005), sugiere que polimorfismos de cambio de amino ácido en la β-tubulina VI y/o polimorfimos en regiones reguladoras de los genes de β-tubulina podrían influir en los efectos del paclitaxel en pacientes.

II. FARMACOGENÉTICA

II.1. Variabilidad interindividual en los efectos de los fármacos

La variación interindividual en los efectos de los fármacos representa uno de los mayores retos para la mejora de la farmacoterapia y la práctica clínica. Esta variación afecta tanto a la eficacia como a las reacciones adversas o ADRs (del inglés *Adverse Drug Reactions*) de los tratamientos farmacológicos.

El porcentaje de pacientes que no responden a fármacos de uso común abarca entre un 20 y un 75% (Kalow 2001). Por otro lado, las reacciones adversas a fármacos son frecuentes y suponen cerca del 7% de todos los ingresos hospitalarios en los países occidentales (Budnitz 2006, Gurwitz 2007, Pirmohamed 2004). Además, las ADRs influyen en la calidad de vida de los pacientes y comprometen la eficacia de las terapias puesto que pueden requerir reducciones de dosis e incluso, en los casos más severos, la suspensión del tratamiento. En función de la eficacia y toxicidad de un determinado fármaco, los pacientes tratados con éste pueden ser divididos en distintos subgrupos dependiendo de los efectos observados: a) pacientes en los que la eficacia del fármaco es alta y sin toxicidades asociadas, b) pacientes con una alta eficacia del fármaco pero que desarrollan toxicidad, c) pacientes en los que el fármaco no es efectivo y sin toxicidad y d) pacientes en los que el fármaco no es efectivo y que desarrollan toxicidades (Figura 4).

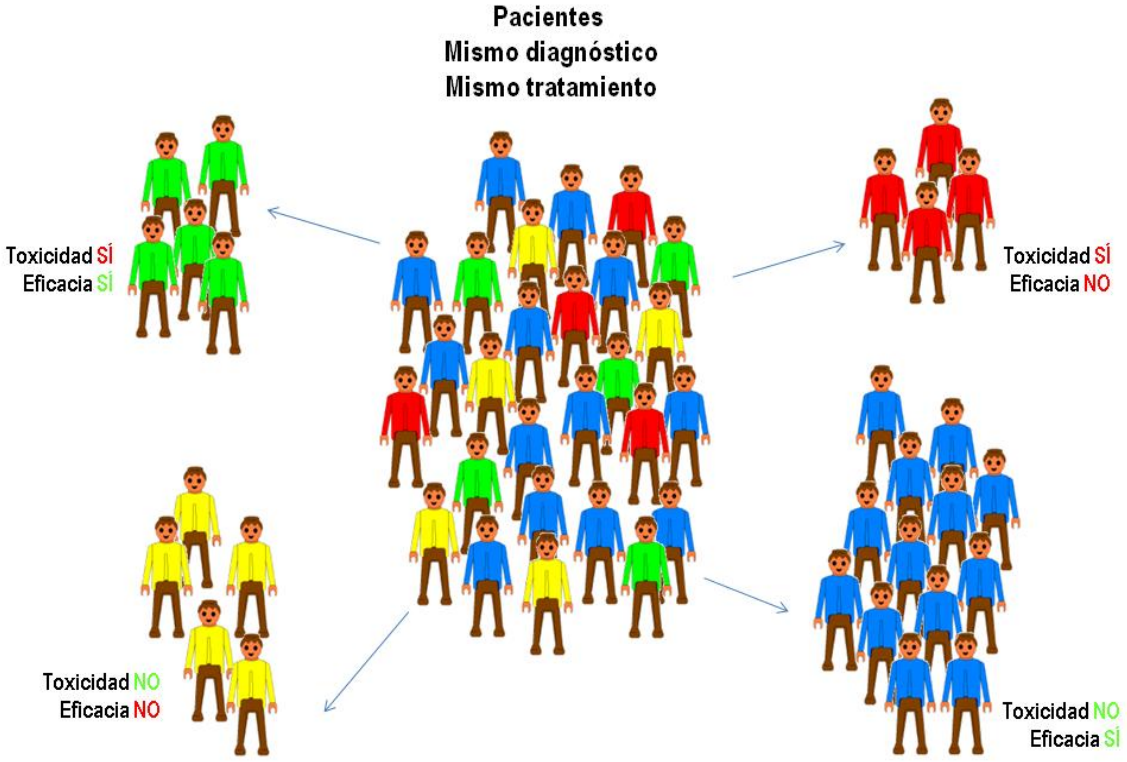

Figura 4. **Variabilidad interindividual de los efectos de los fármacos**. Los pacientes pueden dividirse en cuatro grupos distintos de acuerdo a los efectos del fármaco: respondedores sin toxicidad (en azul), respondedores con toxicidad (en verde), no respondedores sin toxicidad (en amarillo) y no respondedores con toxicidad (en rojo).

En el caso de los fármacos usados en oncología, éstos suelen tener un índice terapéutico bajo (Spear 2001), lo que significa que la dosis que produce el efecto terapéutico deseado y la que produce un efecto tóxico no es muy distinta. Esto se debe a que los fármacos oncológicos suelen ser muy agresivos y, aunque idealmente deberían ser activos exclusivamente en las células tumorales, también mantienen una cierta actividad en las células no-tumorales, por tanto, suelen ser fármacos muy tóxicos. Además, una baja de eficacia de estos tratamientos puede conllevar dramáticas consecuencias para los pacientes. Por ello en oncología, donde la falta de eficacia y las toxicidades severas se encuentran entre los mayores obstáculos para conseguir aumentar la supervivencia de los pacientes, existe una apremiante necesidad de identificar marcadores predictivos de respuesta al tratamiento (Dawood 2009, Ingelman-Sundberg 2008).

Existen múltiples factores que pueden modificar los efectos de un fármaco, tales como factores demográficos y fisiopatológicos. Además de éstos, los factores genéticos también pueden jugar un papel crítico. El impacto de la variabilidad genética en la respuesta y la toxicidad de fármacos ha sido estudiado intensamente en las últimas décadas. Este área de conocimiento, denominada Farmacogenética, ha sido impulsada por la secuenciación del genoma humano y ha experimentado un gran crecimiento desde entonces (Gibson 2007, Nebert 1982, Sjoqvist 1999).

El objetivo de los estudios farmacogenéticos es la identificación de factores genéticos capaces de predecir qué pacientes van a responder de forma óptima a un tratamiento farmacológico, identificando de forma previa al tratamiento individuos con un alto riesgo de desarrollar toxicidades severas y/o falta de eficacia. De esta forma podrían desarrollarse terapias individualizadas, idealmente, proporcionando a cada paciente el fármaco más adecuado a la dosis adecuada, lo cual aumentaría la eficacia terapéutica y disminuiría las toxicidades.

II.2. Variación genética germinal (heredada)

La variación genética está compuesta por las diferencias observadas entre los genomas de los distintos individuos de una especie. Estas variaciones son causadas por mutaciones y recombinaciones en el genoma y su establecimiento y frecuencia en una determinada población son consecuencia de la selección natural y la deriva genética. Se ha estimado que de media, la diferencia en la secuencia del genoma de dos individuos escogidos al azar es de aproximadamente un 0.1% (Shastry 2009). Esta diferencia se debe en su mayoría a los polimorfismos genéticos. Un polimorfismo genético se define como un *locus* en el cual la variante menos frecuente está presente en al menos un 1% de la población; variantes menos comunes se definen como mutaciones o variantes raras (Nebert 2000). La variación genética más común es el polimorfismo de un solo nucleótido o SNP (del inglés *Single Nucleotide Polymorphism*). Este tipo de variante puede localizarse en cualquier lugar del genoma: regiones codificantes de genes, regiones no codificantes o regiones inter-génicas. Se denominan SNPs no-sinónimos a aquellos polimorfismos que causan un cambio de amino ácido en una determinada proteína, y sinónimos a aquellos que se localizan en las regiones codificantes de los genes pero no conllevan

cambios de amino ácido en la proteína. Los SNPs también pueden afectar la transcripción de un gen, la estabilidad de un ARN mensajero o mRNA (del inglés *messenger RNA*), su procesamiento post-transcripcional o *splicing*, la unión de ARNs no codificantes o ncRNAs (del inglés *Non Coding RNAs*), etc., afectando finalmente la cantidad de proteína y por tanto su actividad. Otras formas comunes de variación genética son las repeticiones cortas en tándem o STRs (del inglés *Short Tandem Repeats*) también conocidas como microsatélites, repeticiones en tándem de número variable o VNTRs (del inglés *Variable Number Tandem Repeats*) y polimorfismos de pequeñas inserciones y deleciones (<1 Kb) o in-dels (Feuk 2006, Mills 2006). También existen polimorfismos de ganancia o pérdida de regiones del genoma mayores a 1 Kb, conocidos como variaciones en el número de copias o CNVs (del inglés *Copy Number Variants*) (Conrad 2010, Zhang 2006). Aunque las CNVs no suelen conllevar alteraciones en la secuencia de las proteínas codificadas en el genoma, la presencia de varias copias de un mismo gen puede conducir al incremento en la cantidad de proteína. Por el contrario, deleciones completas o parciales de un gen conducen a menores cantidades de proteína.

Existen múltiples publicaciones que identifican SNPs asociados a una mayor susceptibilidad a desarrollar enfermedades comunes como diabetes (Yasuda 2008), enfermedades coronarias (Willer 2008) o cáncer (Antoniou 2008), entre otras. Más recientemente, también se han descrito asociaciones entre CNVs y el riesgo a desarrollar varias enfermedades humanas (Bae 2010, Lesch 2011, McCarroll 2007, Tam 2009, Wain 2009). Con respecto a los efectos de fármacos, también se han descrito asociaciones entre éstos y SNPs y CNVs (Cheok 2006, Goetz 2007, Hulot 2006, Kirchheiner 2001, Kweekel 2008, Laverdiere 2002, Mallal 2002).

II.3. Variación genética somática (adquirida)

La farmacogenética en el campo de la oncología es especialmente compleja ya que hay dos tipos de genomas a estudio: el genoma del individuo afecto y el genoma del tumor. Tanto la variación genética heredada, intrínseca a todas las células del individuo (variación germinal), como la variación genética adquirida, en las células tumorales (variación somática), pueden conducir a diferencias inter-individuales en los efectos provocados por los fármacos (Figura 5).

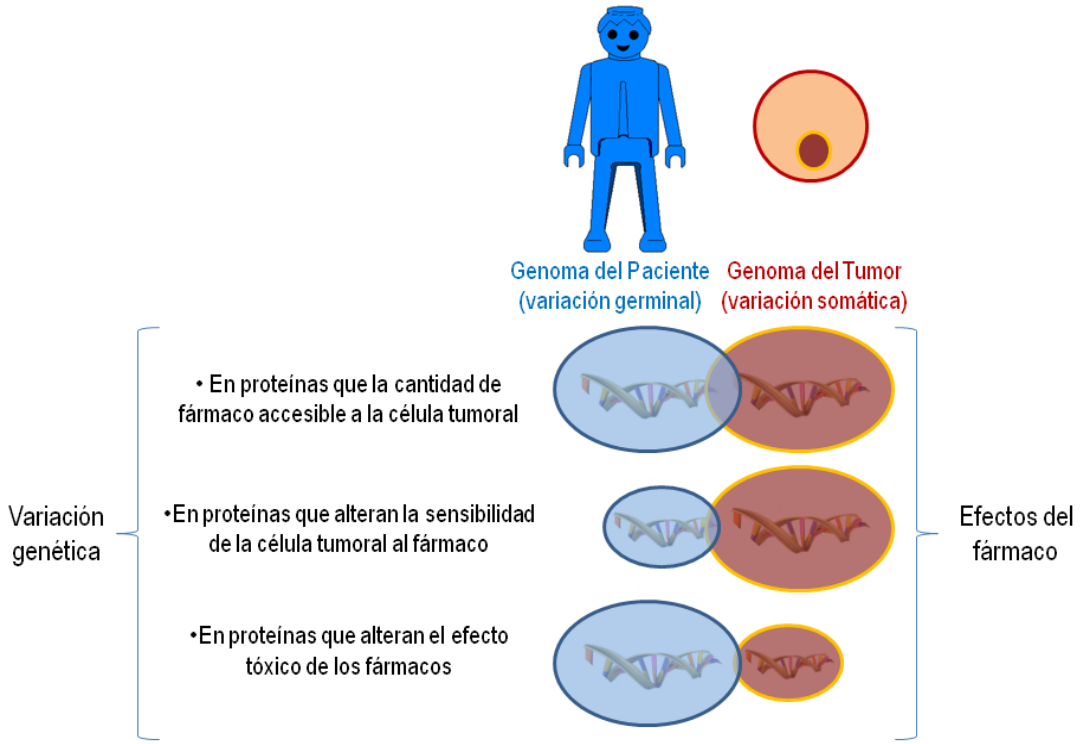

Figura 5. La variación germinal y somática y su influencia en los efectos de los fármacos.

II.4. Estrategias de estudio en Farmacogenética

La farmacogenética fundamentalmente utiliza estudios de asociación para identificar variantes genéticas asociadas a la eficacia/toxicidad de los fármacos. Los estudios de asociación se basan en el descubrimiento de la relación causa-efecto de una variante genética y un determinado fenotipo, tal como un rasgo físico, susceptibilidad a desarrollar una determinada enfermedad o una respuesta a un fármaco. El éxito de estos estudios está supeditado a varios aspectos fundamentales: el reclutamiento de series con suficientes individuos, la frecuencia de los polimorfismos estudiados y el riesgo asociado a la presencia de éstos (Fletcher 2010). Por ejemplo, en estudios de asociación caso-control para la identificación de variantes asociadas a desarrollar ciertas enfermedades, son necesarios 300 pacientes y 300 controles para detectar la asociación de una variante relativamente frecuente (con una frecuencia alélica del 20% en población general), que confiera un riesgo moderado (de 1.5 para los portadores), con un nivel de significación α=0.05 y una potencia del 80%. Los estudios de asociación en farmacogenética se benefician de variantes genéticas con riesgos atribuibles mayores, por tanto, el número adecuado de individuos a estudio podría ser menor.

II.4.a. Estrategias de genes candidatos

La mayoría de los estudios farmacogenéticos que se han llevado a cabo hasta la actualidad se basan principalmente en estrategias de genes candidatos que aprovechan el conocimiento del conjunto

de eventos que transcurren desde la administración de un fármaco hasta su efecto, o lo que es lo mismo, las rutas farmacocinéticas (PK, del inglés *PharmacoKinetics*) y farmacodinámicas (PD, del inglés *PharmacoDynamics*). En particular, la PK comprende los procesos de absorción, distribución, metabolismo y excreción, y describe la concentración en plasma del fármaco y sus metabolitos, y el tiempo que éstos permanecen en él (Dawood 2009). Entre los genes más relevantes para la PK están aquellos que codifican las familias 1-3 de enzimas metabólicas Citocromo P450 (CYP) (Evans 1999, Shimada 1994, Wrighton 1992), las enzimas de conjugación uridina difosfato glucuronosiltransferasas (UGT), sulfotransferasas (ST) y glutation S-transferasas (GST) (Burchell 2003), y los transportadores que median la absorción y bombeo del fármaco: transportadores de aniones orgánicos (OAT), polipéptidos transportadores de aniones orgánicos (OATP), transportadores de cationes orgánicos (OCT), transportadores con sitio de unión de ATP (transportadores ABC, del inglés *ATP Binding Cassette*) que incluyen la glicoproteína P (P-gp) y otras proteínas relacionadas con resistencia a múltiples fármacos (MDRs) (Chan 2004). La PD estudia los efectos bioquímicos y fisiológicos de los fármacos y sus mecanismos de acción que dan lugar a la eficacia terapéutica y/o efectos tóxicos (Dawood 2009). Las dianas terapéuticas de un compuesto se encuentran entre las proteínas más relevantes para la PD.

Los estudios de genes candidatos centrados en las rutas PK y PD se basan en la hipótesis de que variaciones en los genes que codifican las proteínas implicadas en estas rutas podrían afectar el efecto del fármaco. La mayoría de los estudios farmacogenéticos llevados a cabo hasta la fecha han utilizado esta estrategia. Con el tiempo, el conocimiento sobre estas rutas ha aumentado y para muchos fármacos ya se conocen sus enzimas metabolizadoras, sus metabolitos, sus transportadores y sus dianas terapéuticas. Por ello, los estudios de genes candidatos han evolucionado: del estudio de unos pocos genes clave se ha pasado al estudio de todos los genes que codifican los miembros conocidos de las rutas PK y PD de un determinado fármaco (Evans 2004). Además, un único polimorfismo/gen probablemente sea insuficiente para explicar la variabilidad inter-individual en los efectos de muchos fármacos. De este modo, sería la combinación de varios polimorfismos en uno o más genes, de la misma o de distintas rutas, junto con factores clínicos y fisiopatológicos, los que explicarían la variabilidad en los efectos observados en los pacientes. De forma similar a los polimorfismos, también se puede estudiar el impacto de variantes genéticas adquiridas en las células tumorales (variación somática) en el efecto de los fármacos. En este caso, el efecto a estudiar es la eficacia del fármaco, y las variantes se buscan en genes implicados principalmente en la PD del fármaco. En conclusión, los estudios de genes candidatos, han demostrado ser una estrategia válida y eficaz para la identificación de variantes genéticas asociadas a los efectos de los fármacos, si bien están limitados por los conocimientos disponibles sobre el fármaco y sus rutas de PK y PD.

II.4.b. Estrategias de genoma completo

Más recientemente, el desarrollo de plataformas de alto rendimiento de genotipado del genoma completo, capaces de genotipar simultáneamente millones de SNPs, ha permitido la aparición de una

nueva estrategia en los estudios de asociación. Sin basarse en ningún conocimiento biológico previo, estas plataformas permiten estudiar el genoma completo e identificar variantes comunes asociadas con los fenotipos/efectos estudiados. Se conocen como estudios de asociación del genoma completo o GWAS (del inglés *Genome Wide Association Studies*). Esta estrategia es muy útil para el descubrimiento de nuevos genes que no habían sido relacionados con el fármaco pero que son importantes para su efecto (Nelson 2009). Las plataformas de genotipado de alto rendimiento que utilizan estos estudios están compuestas principalmente por SNPs marcadores o tagSNPs (del inglés *Tag Single Nucleotide Polymorphisms*), representativos de una determinada región del genoma con un alto desequilibrio de ligamiento. Se basan en datos disponibles del proyecto Hapmap (www.hapmap.org), y representan la variación común a lo largo de todo el genoma. Por lo tanto, el genotipado de estos tagSNPs proporciona información sobre todos los polimorfismos en desequilibrio de ligamiento con éstos, aunque no estén representados directamente en la plataforma de genotipado. Debido al amplio número de variantes estudiadas en estas plataformas, el número de SNPs falsamente asociados al fenotipo (falsos positivos) es muy alto. Esta limitación suele ser contrarrestada con estudios de replicación/validación en series de pacientes independientes. Otra limitación de los estudios de asociación del genoma completo es que muchas de las asociaciones identificadas son complicadas de evaluar siendo difícil encontrar la variante causal. Muchos de los SNPs genotipados en estas plataformas se localizan en regiones no codificantes, ya sean regiones intergénicas o intrones. Por tanto, muchas de las asociaciones identificadas corresponden a polimorfismos de este tipo. Es por ello que en la era post-GWAS que acaba de comenzar, una de las tareas principales es la identificación de las variantes causales responsables de las asociaciones observadas. Esta tarea se presume mucho más complicada de que lo que se esperaba con el nacimiento de los GWAS.

En los últimos años, el número de GWAS en farmacogenética ha aumentado. Muchos casos han servido para confirmar asociaciones previamente identificadas mediante estrategias de genes candidatos: por ejemplo, variantes de los genes *VKORC1* y *CYP2C9* y la variabilidad en la dosis de warfarina (Cooper 2008, Takeuchi 2009) o variantes del gen *CYP2C19* y la respuesta a clopidogrel (Shuldiner 2009). En otros casos, los GWAS han servido para identificar nuevas asociaciones que posiblemente nunca habrían sido encontradas mediante los estudios de genes candidatos: por ejemplo, la variante *HLA-B*5701* y la susceptibilidad a daño hepático causado por flucloxacilina (Daly 2009). Hasta ahora en los GWAS farmacogenómicos sólo se han detectado variantes genéticas con efectos fuertes, con un riesgo asociado de al menos cuatro veces mayor al de los individuos no portadores de las variantes de riesgo (Daly 2010). Esto se debe en parte a la dificultad de conseguir series amplias de pacientes con datos clínicos asociados. Hay que tener en cuenta que, a diferencia de los estudios de susceptibilidad en cáncer, donde básicamente sólo se necesitan casos con un mismo diagnóstico y controles, en los estudios de farmacogenética son necesarios pacientes tratados de forma homogénea y en los que se haya recogido la eficacia y toxicidad del fármaco de forma detallada y uniforme. Además, el análisis de los datos de los

estudios de farmacogenómica es complicado debido a la evaluación, a veces heterogénea y subjetiva, de los fenotipos de respuesta y toxicidad.

Al igual que en el caso de los estudios de genes candidatos, también se pueden llevar a cabo estudios de genoma completo con las variantes somáticas adquiridas en las células tumorales. En este caso el genoma a estudio no es el del individuo, sino el de la célula tumoral y las variantes adquiridas afectarán principalmente a la eficacia del fármaco. El desarrollo de nuevas tecnologías de secuenciación masiva englobadas en lo que se ha denominado NGS (del inglés *Next Generation Sequecing*), permiten secuenciar el genoma completo de un individuo o de un tumor, e identificar variantes genéticas funcionales y responsables del fenotipo a estudio. El abaratamiento progresivo de la tecnología necesaria para este tipo de secuenciación y la accesibilidad a las herramientas computacionales necesarias para su análisis, hacen del NGS la plataforma con más proyección presente y futura. Existen ya varios estudios basados en esta tecnología que abalan su utilidad en la identificación de variantes germinales y somáticas responsables de diversas patologías (Banerji 2012, Comino-Mendez 2011, Lohr 2012). Es sólo cuestión de tiempo que aparezcan nuevos estudios farmacogenómicos que utilicen esta tecnología (Ellis 2012).

II.5.Traslación de la Farmacogenética a la clínica: presente y futuro

La farmacogenética ha conducido a importantes avances en el estudio de los mecanismos genéticos que subyacen a las diferencias interindividuales observadas en los pacientes tratados con determinados fármacos. La farmacogenética tiene un papel especialmente importante en los tratamientos oncológicos, donde existe una imperiosa necesidad de encontrar marcadores predictivos. Gracias a diversas estrategias, como estudios de genes candidatos y GWAS, se han identificado marcadores que se utilizan en la práctica clínica habitual. En este sentido, ya existen ejemplos de test farmacogenéticos para una variedad de fármacos antineoplásicos que son parte de la atención oncológica rutinaria (Tabla 2). Estos test detectan variantes genéticas en el genoma tumoral (variantes somáticas) o en el genoma de individuo (variantes germinales) que afectan la farmacodinámica o farmacocinética del fármaco (Wang 2011).

Aún así, el uso de test genéticos como fuente de información para tomar decisiones respecto a los tratamientos farmacológicos aún no está muy extendido. La traslación a la clínica está siendo lenta y esto se debe a varias razones. El aspecto más importante es la necesidad de establecer la utilidad clínica y el coste-beneficio de los biomarcadores identificados de forma objetiva. En ausencia de esta evidencia, será difícil convencer a los organismos pertinentes de que provean financiación para el establecimiento rutinario de estos test farmacogenéticos, lo que los convertirá en inaccesibles para la gran mayoría de los pacientes. Desafortunadamente, no existe consenso sobre las evidencias necesarias que determinarían tal utilidad clínica.

Es evidente que existe la necesidad de llevar a cabo ensayos clínicos que estratifiquen de manera prospectiva a los pacientes en función de las variaciones genéticas relevantes. Igualmente, existe

la necesidad de establecer consorcios internacionales para llevar a cabo estudios colaborativos que incluyan un gran número de sujetos. Toda esta investigación necesita ser financiada y, en el caso de fármacos con patente, las empresas farmacéuticas que los comercializan podrían estar interesadas en promoverlas. En el caso de fármacos sin patente, la obtención del soporte económico necesario para llevar a cabo estas investigaciones es más difícil. Es también esencial el desarrollo de algoritmos sencillos que ayuden a los clínicos a la interpretación y uso de los datos genéticos, así como una formación específica para ellos. En esta línea, la FDA está modificando los prospectos de los fármacos para los que se ha descrito claramente una asociación farmacogenética con un biomarcador (Tabla 2).

Por otro lado, la farmacogenética está siendo una herramienta muy útil en la investigación y desarrollo de nuevos fármacos antineoplásicos. En particular, la investigación en agentes de unión a microtúbulos está ayudando a identificar los mecanismos asociados a su eficacia y a su toxicidad. El futuro de la investigación en los agentes de unión a microtúbulos pasa necesariamente por la consecución de varias metas: 1) aumentar la especificidad de los fármacos sobre el tejido tumoral, 2) reducir las toxicidades asociadas y 3) reducir los mecanismos de resistencia (Dumontet 2010).

Los agentes de unión a microtúbulos permanecen, desde prácticamente medio siglo, como uno de los aliados más importantes en la lucha contra el cáncer. Entre ellos, el paclitaxel, objeto de este trabajo de tesis, es un componente clave del tratamiento de numerosos tipos de tumores sólidos. El estudio de los mecanismos involucrados en la respuesta a este fármaco es esencial para optimizar su terapia, pero no menos importante es la estratificación de los pacientes, pudiendo identificar a aquellos con un alto riesgo de ser resistentes al tratamiento o de desarrollar toxicidades severas. En esta Tesis Doctoral hemos aportado nuevos datos con respecto a la expresión, regulación y variación genética de los isotipos de β-tubulina, la diana terapéutica del paclitaxel. Estos conocimientos nos han permitido proponer diversos marcadores predictivos de la eficacia y toxicidad de este fármaco. Además, utilizando una aproximación de genoma completo hemos identificado nuevos marcadores de la neuropatía inducida por paclitaxel en genes hasta ahora no relacionados con el fármaco. De ser validados, estos marcadores podrían tener de una gran utilidad clínica, ayudando a una individualización de la terapia con paclitaxel.

Tabla 2. Fármacos antineoplásicos aprobados por la FDA con etiquetado sobre biomarcadores farmacogenómicos en el área de oncología.
(www.fda.gov/Drugs/ScienceResearch/ResearchAreas/Pharmacogenetics/ucm083378.htm).

Fármaco	Biomarcador
Arsenic Trioxide	PML/RARa
Brentuximab Vedotin	CD30
Busulfan	Ph Chromosome
Capecitabine	DPD
Cetuximab (1)	EGFR
Cetuximab (2)	KRAS
Cisplatin	TPMT
Crizotinib	ALK
Dasatinib	Ph Chromosome
Denileukin Diftitox	CD25
Erlotinib	EGFR
Exemestane	ER &/ PgR receptor
Fulvestrant	ER receptor
Gefitinib	EGFR
Imatinib (1)	C-Kit
Imatinib (2)	Ph Chromosome
Imatinib (3)	PDGFR
Imatinib (4)	FIP1L1-PDGFRa
Irinotecan	UGT1A1
Lapatinib	Her2/neu
Letrozole	ER &/ PgR receptor
Mercaptopurine	TPMT
Nilotinib (1)	Ph Chromosome
Nilotinib (2)	UGT1A1
Panitumumab (1)	EGFR
Panitumumab (2)	KRAS
Pertuzumab	Her2/neu
Rasburicase	G6PD
Tamoxifen	ER receptor
Thioguanine	TPMT
Tositumomab	CD20 antigen
Trastuzumab	Her2/neu
Vemurafenib	BRAF

OBJETIVOS

OBJETIVOS

El objetivo principal de esta tesis fue la identificación de marcadores biológicos asociados con la eficacia y la toxicidad del paclitaxel. Para ello se han utilizado dos estrategias. Por una parte, estudiamos la expresión, regulación y variación genética de la diana terapéutica de este fármaco, la β-tubulina y sus correspondientes isotipos. La segunda estrategia, consistió en buscar marcadores genéticos asociados con la neuropatía causada por el paclitaxel mediante una aproximación de GWAS. En conjunto, estos datos podrían proporcionar una base para la individualización de la terapia con paclitaxel. Estos objetivos generales se desglosan en los siguientes objetivos específicos:

1. Determinar los patrones de expresión de los 8 isotipos humanos de β-tubulina en tejidos tumorales y no tumorales.

2. Establecer si los microARNs, en concreto la familia miR-200, regulan la expresión proteica de los isotipos de β-tubulina. Si es así, determinar si los microARNs influyen en la respuesta y supervivencia de pacientes con cáncer de ovario avanzado tratados con paclitaxel/ carboplatino.

3. Identificar variantes genéticas codificantes en la β-tubulina VI y determinar su posible efecto en la mielosupresión causada por el paclitaxel.

4. Identificar variantes genéticas que regulen la expresión de la β-tubulina IIa y determinar su posible efecto en la neuropatía inducida por paclitaxel.

5. Identificar marcadores genéticos asociados con la neuropatía del paclitaxel utilizando un estudio de asociación del genoma completo (GWAS).

ARTÍCULOS

ARTÍCULO 1: Tumoral and tissue-specific expression of the major human β-tubulin isotypes.

Autores: Leandro-García LJ, Leskelä S, Landa I, Montero-Conde C, López-Jiménez E, Letón R, Cascón A, Robledo M, Rodríguez-Antona C.

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Resumen: Las β-tubulinas, componentes fundamentales de los microtúbulos, se encuentran codificadas por una familia multigénica que produce proteínas ligeramente distintas con patrones de expresión complejos. Varios fármacos antineoplásicos ampliamente utilizados basan su actividad en la unión a la β-tubulina, alterando la dinámica de los microtúbulos y bloqueando la división celular. La expresión de estas dianas terapéuticas en las células no-tumorales y tumorales podría ser de crucial importancia para la respuesta a estos fármacos, pero hasta la fecha la caracterización de los patrones de expresión de las β-tubulinas ha sido pobre. En este estudio hemos aplicado la técnica cuantitativa de RT-PCR para determinar con precisión la expresión de ARNm de los ocho isotipos humanos de β-tubulina que codifican las clases I, IIa, IIb, III, IVa, IVb, V y VI en 21 tejidos no tumorales y en 79 muestras de tumores pertenecientes a siete tipos de cáncer. En los tejidos no tumorales encontramos que el patrón de expresión y la contribución de los diferentes isotipos al contenido total de β-tubulina variaron para cada tejido. En general, *TUBB* (I), *TUBB2C* (IVb) y *TUBB6* (V) fueron isotipos ubicuos, *TUBB1* (VI) fue un isotipo específico de células hematopoyéticas y *TUBB2A* (IIa), *TUBB2B* (IIb), *TUBB3* (III) y *TUBB4* (IVa) tuvieron una alta expresión en el cerebro. En los tejidos tumorales, la mayoría de los isotipos tuvieron una expresión alterada y diferente, dependiendo del tipo de tumor o de características específicas de los tumores. En la mayoría de los tumores la expresión de *TUBB3* aumentó mientras que la de *TUBB6* disminuyó. En conjunto, podemos concluir que la compleja distribución de los isotipos de β-tubulina en los tejidos normales podría ayudar a explicar el perfil de toxicidades de los fármacos de unión a microtúbulos. Además, diferencias en la expresión tumoral de los isotipos podría contribuir a las diferencias en la eficacia de estos fármacos en los pacientes.

Aportación personal: LJ Leandro-García participó en el diseño del estudio, llevó a cabo el procesamiento de las muestras de tejido y recopilación de sus características, extracción de ARN total, retro-transcripción a ADNc y qPCR. Participó en el análisis de los datos para la cuantificación y el análisis estadístico. Finalmente, LJ Leandro-García participó activamente en la discusión crítica de los resultados y fue el autor principal en la redacción del trabajo para su publicación.

RESEARCH ARTICLE
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Tumoral and Tissue-Specific Expression of the Major Human β-Tubulin Isotypes

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The β-tubulins are microtubule components encoded by a multigene family, which produces slightly different proteins with complex expression patterns. Several widely used anticancer drugs base their activity on β-tubulin binding, microtubule dynamics alteration, and cell division blockage. The expression of these drug targets in tumoral and normal cells could be of crucial importance for therapy outcome, unfortunately, the complex β-tubulin expression patterns have been poorly characterized in human. In this study, we developed a quantitative RT-PCR technique that accurately determines the mRNA expression of the eight human β-tubulin isotypes, encoding class I, IIa, IIb, III, IVa, IVb, V, and VI and applied it to 21 nontumoral tissues and 79 tumor samples belonging to seven cancer types. In the nontumoral tissues, we found that, overall, *TUBB* (I), *TUBB2C* (IVb), and *TUBB6* (V) were ubiquitous, *TUBB1*(VI) was hematopoietic cell-specific, and *TUBB2A* (IIa), *TUBB2B* (IIb), *TUBB3* (III), and *TUBB4* (IVa) had high expression in brain; however, the contribution of the different isotypes to the total β-tubulin content varied for each tissue and had a complex pattern. In tumoral tissues, most isotypes exhibited an altered expression in specific tumor types or related to tumoral characteristics. In general, *TUBB3* showed a great increase in expression while *TUBB6* expression was largely decreased in most tumors. Thus, normal tissues showed a complex β-tubulin isotype distribution, which could contribute to the toxicity profile of the microtubule-binding drugs. In addition, the specific isotypes significantly altered in tumors might represent markers for drug response. © 2010 Wiley-Liss, Inc.

Key Words: β-tubulin, microtubules, isotypes, microtubule-binding drugs

Additional Supporting Information may be found in the online version of this article.

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Introduction

Microtubules are ubiquitous polymers that perform many different functions, being critically involved in mitosis, intracellular transport, asymmetric morphology of neurons, and ciliary and flagellar motility. Microtubules are composed by heterodimers of α- and β-tubulin, which are continuously incorporated and released, rendering microtubules highly dynamic structures [Risinger et al., 2009]. In mammals, there are at least six α- and seven β-tubulin isotypes, encoded by multiple genes that display differential tissue expression [Lopata and Cleveland, 1987; Ludueña, 1998]. The genes are constituted by four exons and three introns, while the nonfunctional pseudogenes lack most or all introns [Berrieman et al., 2004]. The β-tubulin isotypes are well conserved across species and have related amino acid sequences differing primarily within the C-terminal 15–20 amino acids, a region of the protein that is the putative binding site for several microtubule-associated proteins (MAPs) [Sullivan and Cleveland, 1986]. The expression pattern of the different β-tubulin isotypes has been studied in a fragmented manner, mainly using specific antibodies binding to the C-terminal of the different isotypes that cross-reacted between some of the isotypes, in tissues from a variety of mammals (mainly chicken, rat and mouse) and in cell lines of human origin [Cleveland, 1987; Hiser et al., 2006]. Nonetheless, these studies have shown that some β-tubulin isotypes seem constitutive (class I, IVb and maybe V) while other isotypes are restricted to specific tissues (class II, III, and IVa neuronal; class VI haemopoietic) [Sullivan et al., 1986; Wang et al., 1986; Cleveland, 1987; Lopata and Cleveland, 1987; Verdier-Pinard et al., 2005]. However, a comprehensive characterization of the distribution of the β-tubulin isotypes in human tissues has not been performed.

Concerning cancer, several antineoplastic drugs extensively used and effective in a wide range of tumor types base their activity on the alteration of microtubule dynamics (e.g. taxanes and *Vinca* alkaloids). The binding of these drugs to β-tubulin arrests cells in mitosis and finally causes cell death. Class I β-tubulin mutations altering the binding of the drug have been shown to cause acquired resistance in cell lines

[Giannakakou et al., 1997, 2000; Kavallaris et al., 2001; Verrills et al., 2003]; however, in tumors this mechanism does not seem to play a substantial role [Berrieman et al., 2004]. On the other hand, the expression of some β-tubulin isotypes has been shown to change in malignant cells. Although the role of each isotype has not been fully elucidated, several lines of evidence suggest that differential expression of β-tubulin isotypes is involved in anticancer-drug-resistance. More specifically, class III tumoral overexpression has been associated with clinical response to different microtubule-inhibitors [Hasegawa et al., 2003; Mozzetti et al., 2005; Ferrandina et al., 2007, 2006; Seve et al., 2005, 2007a,2007b]. Thus, it is clear that a precise characterization of β-tubulin isotype expression in human normal and tumoral tissues could help us understand the molecular basis of toxicity profiles, drug-sensitivity and resistance in cancer patients treated with microtubule binding drugs and help to customize chemotherapy.

In this study, we present a novel qRT-PCR technique which allows to measure with high accuracy, while avoiding cross-reactions, the human β-tubulin isotypes class I, IIa, IIb, III, IVa, IVb, V, and VI (encoded by *TUBB, TUBB2A, TUBB2B, TUBB3, TUBB4, TUBB2C, TUBB6*, and *TUBB1*, respectively). We used this technique to establish the mRNA expression of these eight human β-tubulin isotypes in 21 nontumoral and 79 tumor samples belonging to seven cancer types, finding complex expression patterns according to the different tissues and tumor type.

Materials and Methods

Human Samples

cDNAs from 20 different nontumoral tissues were obtained from BD Biosciences (human Multiple Tissue cDNA (MTC) panel I and II and human immune system panel). In addition, the study included the following frozen tissues: 10 matched nontumoral/tumoral prostate samples, 4 matched breast nontumoral/tumoral and 23 breast tumor samples, 6 clear cell renal carcinomas, 5 nonsmall cell lung cancer samples, 7 colon adenocarcinomas, 4 ovarian serous carcinomas, and 20 larynx squamous cell carcinomas. Tissue samples were provided by the Tissue Bank Network coordinated by the Molecular Pathology Program of the Spanish National Cancer Centre (CNIO), with the collaboration of the Department of Pathology of several Spanish Hospitals. The main characteristics of the commercial and frozen samples are shown in Table I. All samples were of Caucasian origin and the cancer tissues corresponded to tumors that had not received chemotherapy before the surgery, except for three larynx tumors that received cisplatin.

RNA Isolation and Real-Time Quantitative RT-PCR

RNA was isolated from the frozen tissue using TRI-reagent (Molecular Research Center, Cincinnati, OH). Reverse transcription was performed in a single tube using 1 μg of the total RNA, Superscript II Reverse Transcriptase (Invitrogen, CA) and an oligo dT14 primer following the manufacturer's instructions.

The amounts of the different β-tubulin isotypes mRNA were quantified by real-time PCR with the Sequence Detection System 7900HT (Applied Biosystems, Foster City, CA), using primers designed to be specific for each isotype and probes from the Universal ProbeLibrary Set, Human (Roche). The final concentrations of the primers (shown in Table II) and the probes were 200 nM and 100 nM, respectively, with a final volume of 12 μl. For the amplification reaction, the Universal Master Mix (PE Applied Biosystems) was used and the conditions consisted in an initial step of 95°C for 10 min, followed by 55 cycles of 15 s at 95°C and 1 min at 60°C. For isotypes *TUBB*, *TUBB2B*, and *TUBB2C* the forward primer was located in the 5′UTR; for the rest of the isotypes both forward and reverse primers were located in the coding region. In all cases at least one mismatch provided specificity and avoided cross-reactions. To assess the specificity obtained with these primers and amplification conditions, the 8 β-tubulin isotypes were amplified as described above in five liver and six breast cDNAs, the PCR products of each isotype were pooled together, purified using the PCR Purification Kit (QIAGEN) and sequenced. The sequence obtained for each β-tubulin revealed that only the corresponding isotype was amplified and cross-reactions with other isotypes or pseudogenes was ruled out.

To perform the quantification, standard curves for the different β-tubulin isotypes were generated with serial 1/10 dilutions of the MTC cDNA with the maximal expression in each specific isotype. Normalization was carried out with the internal standard β-glucuronidase (GUS), negative controls were present in all series of PCR reactions and all assays were carried out in triplicates. To obtain comparable amounts of the different β-tubulin isotypes within the same sample, the same fluorescence threshold was used (0.025) for all isotypes' quantification. At this value all reactions were in the initial part of the exponential amplification phase. The cycle at which each sample crossed the threshold (Ct) was recorded for all isotypes and GUS. It was confirmed that the Ct values remained constant from run to run by assessment in two independent experiments. The delta–delta Ct method [Livak and Schmittgen, 2001] was used for the calculation of the different amounts of mRNA: in brief, ΔCt was calculated by subtraction of GUS Ct value to the β-tubulin isotypes Ct values. The ΔCt difference between each sample and a reference sample was calculated (ΔΔCt) and this value was then used in the formula $2^{-\Delta\Delta Ct}$ to calculate the different amounts. For the ΔΔCt calculation to be valid, the amplification efficiencies of the genes under study must be approximately equal [Livak and Schmittgen, 2001]. Using this technique the amplicons of the β-tubulin isotypes and GUS were very similar (less than 130 bp, see Table II) and the PCR primers/conditions were designed using the same criteria, suggesting optimal PCR amplification efficiencies (E) close to 2. This was verified using the equation $E = 10^{[-1/\text{slope}]}$ [Rasmussen, 2001].

Table I. Characteristics of the Human Tissues Used for qRT-PCR

Tissues	Normal/tumoral	Sample type	Number of samples and characteristics
Brain[a]	Normal	cDNA	Pool from 2
Heart	Normal	cDNA	Pool from 3
Kidney	Normal	cDNA	Pool from 5
Lung	Normal	cDNA	Pool from 4
Liver[a]	Normal	cDNA	1
Colon[b]	Normal	cDNA	Pool from 20
Small intestine[c]	Normal	cDNA	Pool from 32
Pancreas	Normal	cDNA	Pool from 15
Testis	Normal	cDNA	Pool from 45
Prostate	Normal	cDNA	Pool from 32
Ovary	Normal	cDNA	Pool from 14
Placenta	Normal	cDNA	Pool from 17
Bone marrow	Normal	cDNA	Pool from 74
Leukocytes	Normal	cDNA	Pool from >100
Spleen	Normal	cDNA	Pool from 3
Lymph node	Normal	cDNA	Pool from 12
Thymus	Normal	cDNA	Pool from 9
Tonsil	Normal	cDNA	Pool from 5
Skeletal muscle	Normal	cDNA	Pool from 8
Fetal liver	Normal	cDNA	Pool from 32 fetuses
Prostate	Normal	Frozen	10 normal matched[d]
Breast	Normal	Frozen	4 normal matched[d]
Renal	Tumoral	Frozen	6 clear cell renal cell carcinomas, grades 2-3
Lung	Tumoral	Frozen	5 non small cell lung cancers
Colon	Tumoral	Frozen	7 adenocarcinomas, T3N0 to T4N2
Prostate	Tumoral	Frozen	10 adenocarcinomas, grades 2–4[d]
Breast	Tumoral	Frozen	27 infiltrating ductal carcinoma, grades 1–3[d]
Ovary	Tumoral	Frozen	4 serous carcinoma, grades 2–3
Larynx	Tumoral	Frozen	20 squamous cell carcinomas, well-poorly differentiated

[a]Whole.
[b]With mucosal lining.
[c]Without mucosal lining.
[d]Matched tumoral/ nontumoral samples.

For normal prostate, both MTC cDNA and 10 frozen samples were available, and the isotype expression patterns were compared. The similar results obtained with these samples indicated that they were comparable (e.g. in all cases 99% of the total β-tubulin consisted on *TUBB2C, TUBB, TUBB2A*, and *TUBB6*, in decreasing abundance order, data not shown).

Statistical Analysis

Data were analyzed using GraphPad InStat version 3.00 for Windows 95 (San Diego, CA). The method of Kolmogorov-Smirnov was used to test for normality, and when it indicated normality, parametric tests were used. Differences were considered significant when *P*-values were less than 0.05.

Results

Quantitative RT-PCR Measurement of the β-Tubulin Isotypes mRNA in Nontumoral Samples: Tissue-Specific Expression of the Human β-Tubulin Isotypes

Different public databases were used to determine the most abundant relevant human β-tubulin isotypes (using expression levels among different tissues as the criteria for selection, e.g. GeneNote http://bioinfo2.weizmann.ac.il/cgi-bin/genenote/GN_search.pl and CGAP http://cgap.nci.nih.gov/). In consequence, eight different isotypes encoded by the genes *TUBB* (class I), *TUBB1* (VI), *TUBB2A* (IIa), *TUBB2B* (IIb), *TUBB2C* (IVb), *TUBB3* (III), *TUBB4* (IVa), and

Table II. Primer Sequences and Labeled Probes Used for Quantitative RT-PCR

Gene name	Protein name	Accession number	Forward primer[a]	Position[b]	Reverse primer	Position[b]	Probe (5′–3′)	Protein length (bp)
TUBB (HM40)	I	NM_178014	atacctgaggcgagcaaaa	92–111	ctgatcacctcccagaacttg	181–201	UPL #64[c]	110
TUBB1 (Hβ1)	VI	NM_030773	ggatgcgtgaaattgtccat	268–287	agtcgatcccgtgttcctc	345–363	UPL #14	96
TUBB2A (Hβ9[c])	IIa	NM_001069	aaatatgtacctcgggccatc	257–278	gttattccggctccactct	366–386	UPL #50	129
TUBB2B (Hβ9)	IIb	NM_178012	aggacggacagacccagac	43–61	ctgatgacctcccaaaacttg	148–168	UPL #79	126
TUBB2C (Hβ2)	IVb	NM_006088	ttgtctacttcctcctgcttcc	61–82	ctgatcacctcccaaaacttg	156–176	UPL #70	116
TUBB3 (Hβ4)	III	NM_006086	gcaactacgtgggcgact	166–183	cgaggcacgtacttgtgaga	231–250	UPL #78	85
TUBB4 (Hβ5)	IVa	NM_006087	ccggacaacttcgtgtttg	366–384	acagcgtccaccagctct	437–454	UPL #61	89
TUBB6	V	NM_032525	aggctacgtgggagactcg	147–165	gccctgggcacatatttct	215–233	UPL #78	87
GUS		NM_000181	gaaaatacgtggttggaggctcatt	1809–1834	ccgagtgaagatccccttttta	1888–1909	FAMccagcactctcgtcggtgactgttca3BQ1	101

[a]Primer sequences are shown 5′–3′.
[b]The position of the oligonucleotides is referred to the first nucleotide of the mRNA sequence of the corresponding Accession number.
[c]Universal Probe Library (Roche).

TUBB6 (V), were selected for the mRNA quantification technique (accession numbers for each isotype are shown in Table II).

The mRNA expression of the selected β-tubulin isotypes was assessed by using the quantitative RT-PCR technique described in Materials and Methods in 21 nontumoral human tissues. As shown in Fig. 1, the tissues with the highest β-tubulin expressions were as follows: thymus for *TUBB*; peripheral blood leukocytes for *TUBB1*; brain for *TUBB2A, TUBB2B, TUBB3*, and *TUBB4* and heart for *TUBB2C* and *TUBB6*. When comparing the expression of each isotype among the tissues it was evident that *TUBB2A*, *TUBB2B*, *TUBB3*, and *TUBB4* were neuronal-specific isotypes showing much lower expression in all other tissues. *TUBB1* expression was hematopoietic cell-specific with high expression in leukocytes, bone marrow, and fetal liver. *TUBB*, *TUBB2C*, and *TUBB6* were ubiquitously distributed, although *TUBB6* was more important in heart and *TUBB2C* in both heart and testis. The total β-tubulin content, taking into account the eight isotypes here studied, was found maximal for brain, followed by heart and testis (data not shown). These results are similar to previous reports and are in accordance to the key importance of microtubules for these tissues functions.

With respect to the percentage that each isotype represented in each of the 21 nontumoral tissues (Fig. 2a), we found that *TUBB* and/or *TUBB2C* were the major isotypes in all tissues except for brain. Ovary, lymph node, thymus and fetal liver had *TUBB* as the major isotype. Testis expressed mainly *TUBB2C* and very little *TUBB* (more than 50-fold difference) and kidney, heart, skeletal muscle, and lung had more than 3-fold greater levels of *TUBB2C* than *TUBB*, and could be considered mainly *TUBB2C* expressors. On the other hand, in brain, the expression of most isotypes was very different from all of the other tissues studied, and neither *TUBB2C* nor *TUBB* were the major β-tubulin isotypes (Fig. 2a, first bar). Leukocytes expression pattern was also atypical with *TUBB1* being the most abundant isotype (Fig. 2a, eighth bar).

With respect to other isotypes, *TUBB2A* was also present at relevant amounts in a large number of tissues. Its highest expression occurred in brain, where it represented 30% of all β-tubulins, followed by pancreas, liver, small intestine, breast, kidney, prostate, and placenta where its expression ranged between 22 and 11%. *TUBB4* was the major brain isotype (46%), and it also had an important contribution in spleen and testis (representing 13 and 10% of the β-tubulins, respectively). *TUBB1* was the major isotype in leukocytes (50% of all β-tubulins) and it was also expressed in hematopoietic tissues such as fetal liver, bone marrow and spleen, (9, 6, and 3%, respectively), while in the rest of the tissues *TUBB1* represented less than 0.8%. Maximal *TUBB6* expression occurred in breast and lung with 11 and 10% of total β-tubulins, respectively.

The clustering of the isotypes according to their tissue specific expression showed that brain had an expression pattern very different from the other tissues (Fig. 2b). The hematopoietic tissues bone marrow, fetal liver, and spleen clustered

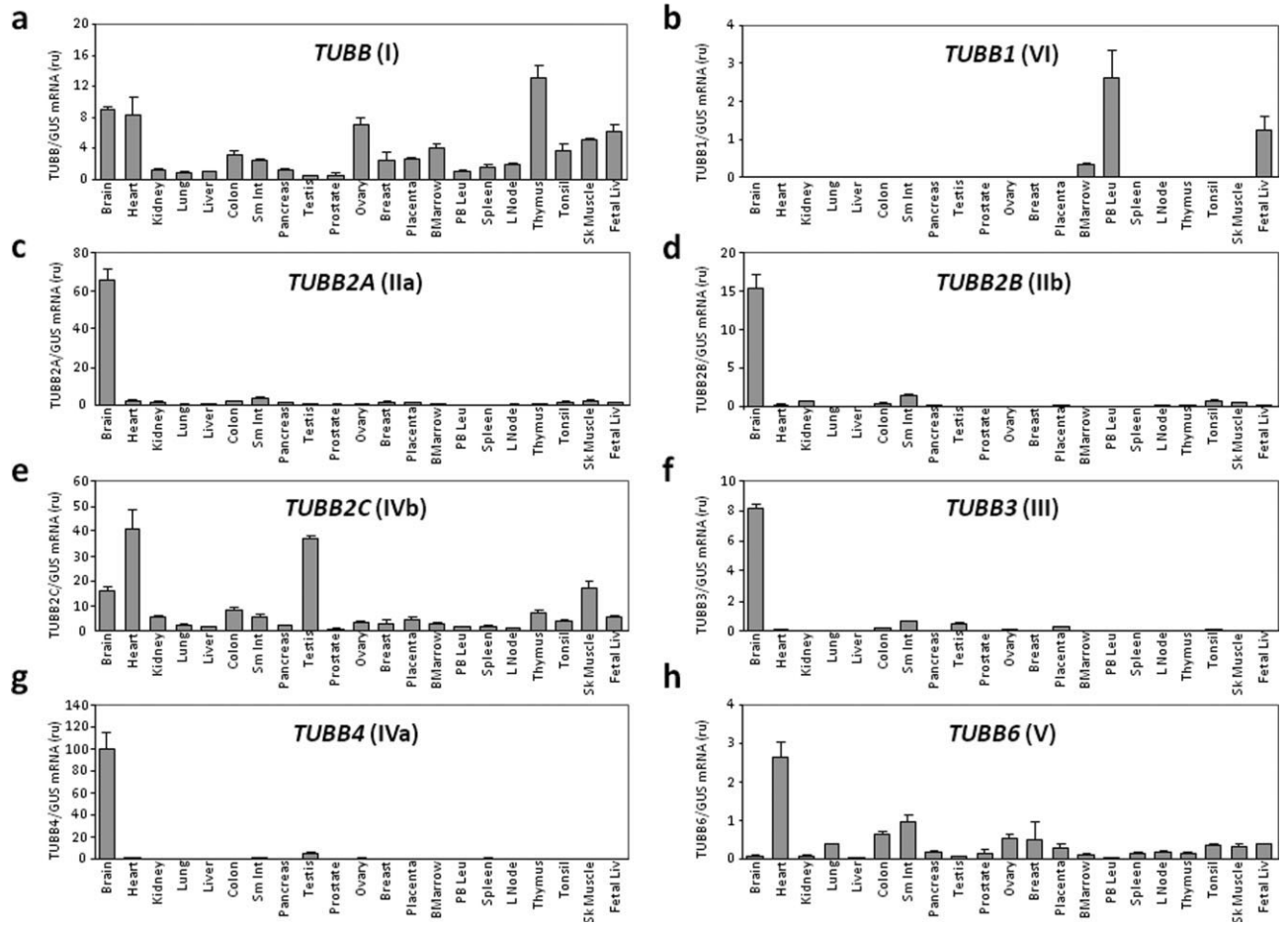

Fig. 1. Expression of eight different human β-tubulin isotypes in human nontumoral tissues. The mRNA content of the β-tubulin isotypes *TUBB, TUBB1, TUBB2A, TUBB2B, TUBB2C, TUBB3, TUBB4*, and *TUBB6* (corresponding to class I, VI, IIa, IIb, IVb, III, IVa, and V isotypes, respectively) was measured by quantitative RT-PCR in 21 normal human tissues, as described in Materials and Methods section. All tissues corresponded to adults except for one fetal sample (fetal liver). The amount of the β-tubulin isotype's mRNA was normalized with the mRNA content of GUS in each sample. The results are referred to the isotype and tissue with the highest β-tubulin expression (*TUBB4* in brain) and represent the mean ± standard deviation.

together, and the same arm contained other tissues related with lymphocyte maturation and accumulation (thymus and lymph nodes); however, ovary, being an unrelated tissue, was also in this arm. The rightmost arm of the cluster contained most of the digestive system organs. With respect to the genes, the ubiquitous *TUBB, TUBB2C*, and *TUBB6* clustered together, as well as the neuronal-specific *TUBB2A, TUBB4, TUBB2B*, and *TUBB3,* whereas the expression pattern of *TUBB1* was the most divergent, similarly to Fig. 1 results.

Human β-Tubulin Isotypes mRNA Expression in Tumoral Samples and Comparison With Nontumoral Tissues

The mRNA contents of the eight selected β-tubulin isotypes were also analyzed in 79 cancer samples corresponding to seven different tumor types (kidney, lung, colon, prostate, ovary, breast, and larynx; see Table I for details). The percentages that each isotype represented in the different tumors are shown in Fig. 2c. *TUBB* was the most abundant isotype in most tumors, followed by *TUBB2C, TUBB2A, TUBB6*, and *TUBB3* (with 47, 38, 8.9, 3.1, and 2.2%, respectively) and with *TUBB4, TUBB2B*, and *TUBB1* levels below 0.5% of the total β-tubulin. Comparing with the nontumoral tissue, the isotype that had the greatest tumoral expression increase was *TUBB3*, which showed a 43- and 71-fold change in breast and lung cancers, respectively (compare *TUBB3* portions in Fig. 2c 2nd and 5th bars with Fig. 2a 11 th and 14th bars), even though *TUBB3* represented in average less than 5% of total tumoral β-tubulin content. *TUBB* also showed a considerable overexpression in kidney and lung tumors (3.8- and 5.9-fold increase). In contrast, *TUBB6* expression was largely decreased in all tumors except for kidney, and *TUBB2C* expression decreased in most tumors.

Since several samples of each tumor type were available, we also studied the variation in the expression among the different tumoral samples. In Fig. 3, the percentages that each isotype represented in the different types of tumors is shown. *TUBB* (Fig. 3a) was the most abundant isotype followed by *TUBB2C* (Fig. 3c), in general mimicking the

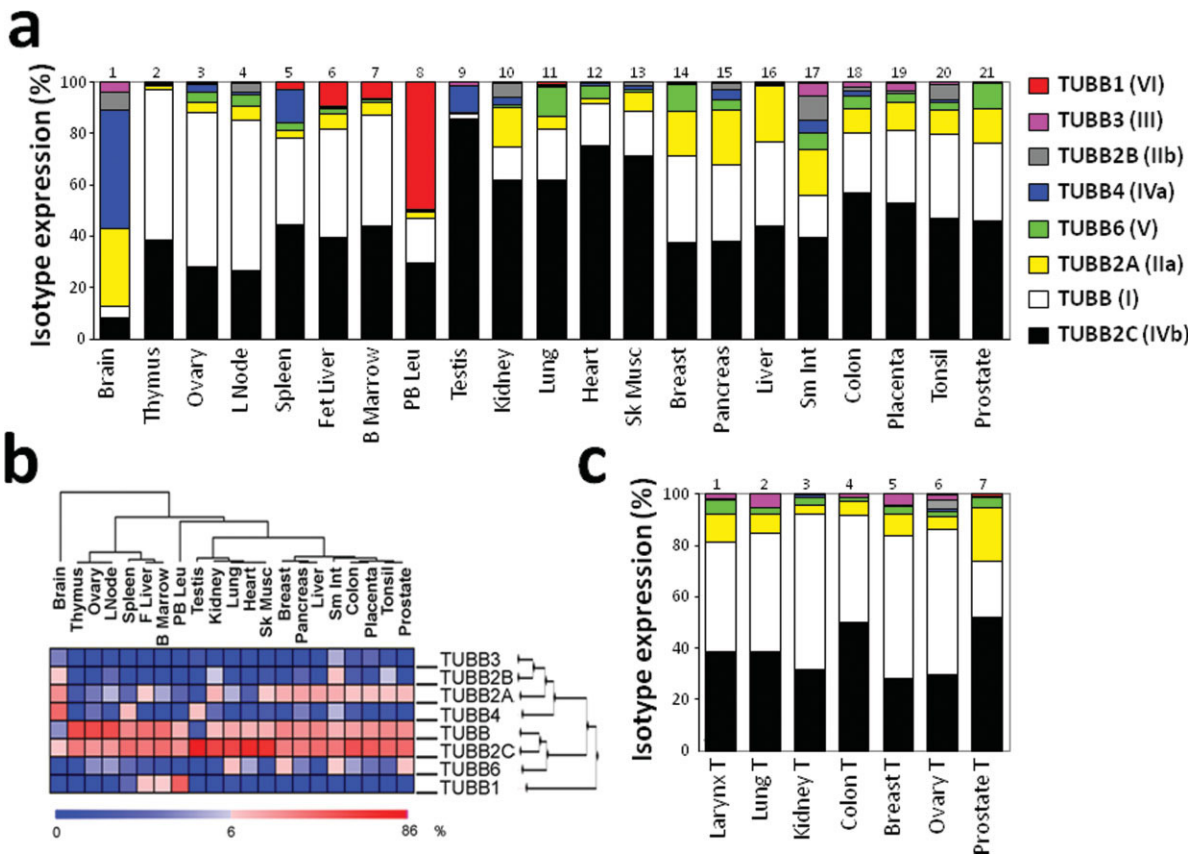

Fig. 2. Relative β-tubulin isotypes mRNA expression in nontumoral and tumoral tissues. (**a**) The mRNA content of the eight isotypes was measured for each nontumoral tissue and the fraction that each isotype represented was calculated and represented in bar graphs. The mRNA quantity of each isotype was calculated as described in Materials and Methods section. (**b**) Unsupervised clustering of the tissue specific relative expression data for the β-tubulin isotypes in nontumoral tissues was performed using the hierarchical Furthest Neighbor (complete linkage) analysis from the GEPAS 4.0 software (http://gepas.bioinfo.cipf.es/) using as distance for gene clustering the correlation coefficient function (offset of 0) c. The percentage of each β-tubulin isotype in different tumors is shown as the mean isotype mRNA expression of each tumor type. The number of samples for each tumor type were 6 kidney, 5 lung, 7 colon, 10 prostate, 4 ovary, 27 breast, and 20 larynx tumoral samples.

normal tissue distribution. *TUBB* maximum and minimum levels occurred in an ovary and a prostate tumor, respectively, with 80 and 12% of the total β-tubulin, while for *TUBB2C* maximum levels (78%) corresponded to a breast sample and the minimum levels (7%) to an ovary tumor. *TUBB3* (Fig. 3d) was the isotype with the largest variation and with levels that reached up to 18, 16, 7.5, and 4.9% of the total β-tubulin in breast, lung, ovary, and larynx tumor samples, respectively. *TUBB2A* (Fig. 3b) and *TUBB6* (Fig. 3e) maximum levels corresponded to breast samples (49 and 14%, respectively).

We then compared the expression of the most relevant tumoral isotypes in the matched nontumoral/ tumoral samples (10 prostate and 4 breast cases, see Table I), we found that *TUBB* and *TUBB6* expression was significantly decreased in prostate tumors ($P = 0.06$ and 0.005, respectively; Supp. Info. Figs. 1a and 1e). In breast tumors only, *TUBB2A* showed a significantly different expression when compared to the matched normal samples (4-fold decreased tumoral expression, $P = 0.0002$; Supp. Info. Fig. 1b). Three of the four matched breast tumoral samples showed increased *TUBB3* expression, however, probably due to the small number of samples analyzed, the results were not significant, despite an average of 1000-fold *TUBB3* tumoral increase.

We then determined whether the expression of the different isotypes could be associated to a specific tumor subtype or differentiation degree in breast and larynx tumors (for which we had the largest number of cases: 27 and 20, respectively). Regarding breast cancer, *TUBB2C* showed significantly higher expression in the more aggressive ER negative tumors than in ER positive ones ($P = 0.008$; Fig. 4). *TUBB* showed a similar tendency, although the differences did not reach significance and *TUBB3* had in average a higher expression in ER negative tumors, and the three tumors with the highest expression corresponded to this type, although the differences were not significant (Supp. Info. Fig. 2). Regarding larynx tumors, none of the isotypes analyzed showed a significantly different expression between the 15 well differentiated and the 5 moderately to poorly differentiated tumors or between the 3 tumors that received cisplatin before the surgery and the 17 tumors that did not (data not shown).

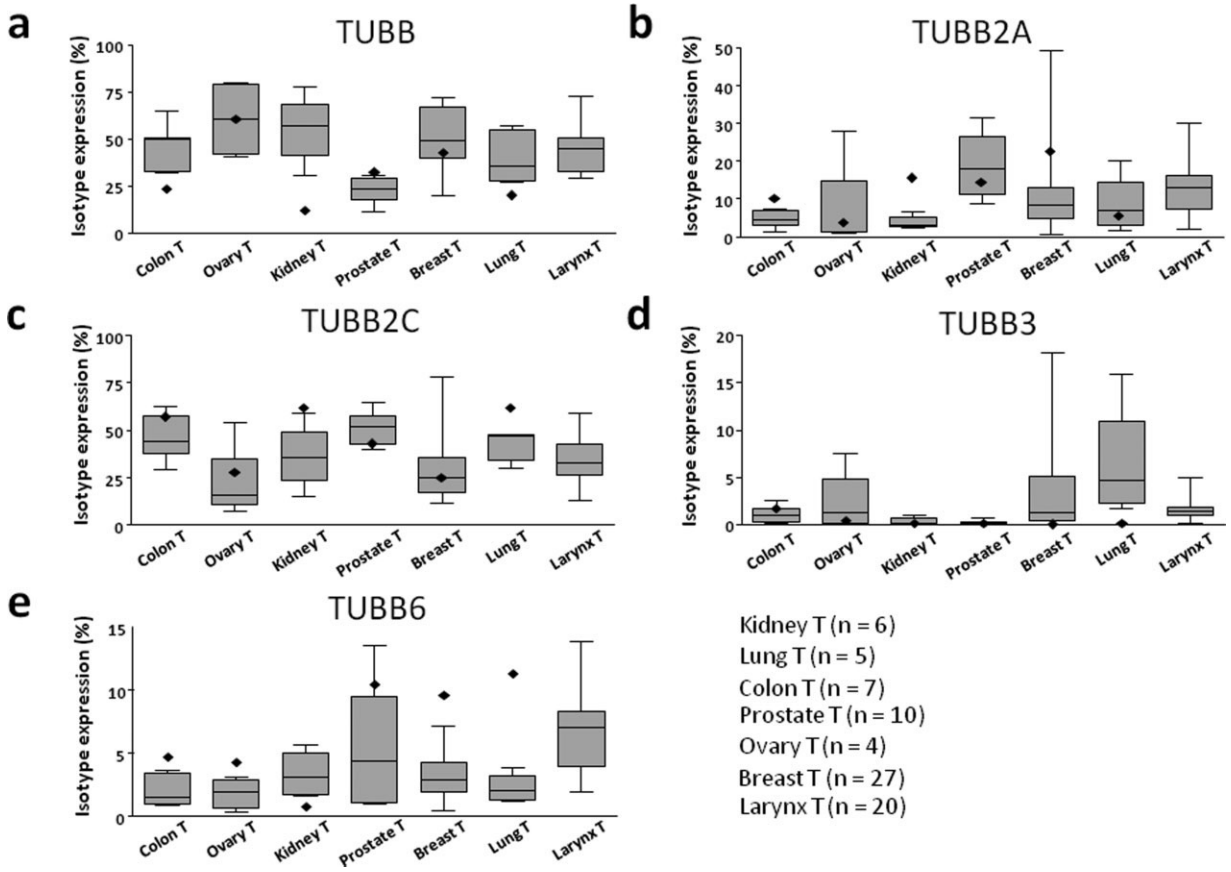

Fig. 3. Tumoral variation in β-tubulin isotypes mRNA expression. The variation in the percentage of each β-tubulin isotype (calculated using the eight different isotypes listed in Table II) among the different tumors is shown in a box plot. The boxes showing the interquartile range, the horizontal line representing the median value for each group, and the whiskers extending to the minimum and maximum values. The diamonds show the expression of the corresponding nontumoral tissue. The number of samples of tumor type are shown at the bottom right.

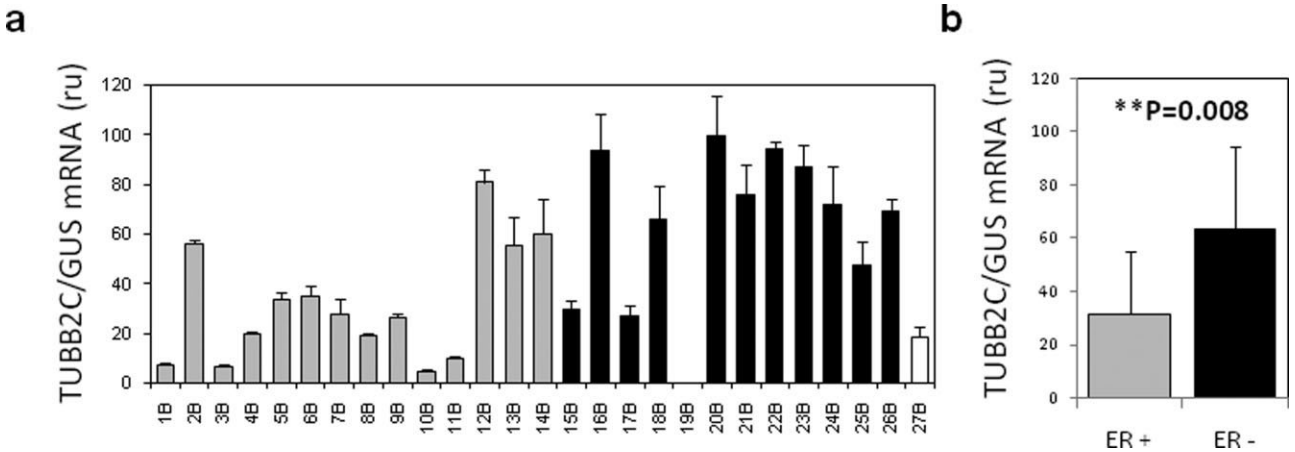

Fig. 4. Expression of *TUBB2C* mRNA in human breast tumors with different estrogen receptor (ER) status. (**a**) The mRNA amounts of *TUBB2C* in 27 breast tumors are shown. (**b**) *TUBB2C* mRNA average values. The mRNA expression of ER positive tumors was statistically significant different from that of ER negative tumors ($P = 0.008$). The gray bars correspond to ER positive tumors, the black bars to ER negative tumoral samples and the white bar to a tumoral sample with unknown ER status.

Discussion

β-tubulins play a crucial role in mitosis and are the targets of several widely used anticancer drugs, however, there is scarce information concerning the β-tubulin isotypes expression in nontumoral and tumoral tissues. Whether this could have an impact on the microtubule binding agents' efficacy and toxicity it is still unknown. In this study, we have

developed a real-time quantitative RT-PCR technique able to accurately determine the mRNA expression of the eight most abundant human β-tubulin isotypes. This technique is able to detect the eight isotypes without cross reactions (i.e. class IVa/IVb and also class IIa/IIb proteins share the same C-terminals, resulting in antibodies that cannot differentiate these isotypes). This technique was used to establish the β-tubulin mRNA expression in a large number of nontumoral and tumoral tissues.

Quantitatively inferring β-tubulin protein levels from mRNA should be done with caution. Nevertheless, general conclusions can be drawn based on mRNA data (e.g. lack of mRNA results in lack of protein, and mRNA increases/decreases in tumoral tissues will likely result in alterations at protein level). This is further supported by good correspondence between β-tubulin mRNA and protein levels [Shalli et al., 2005; Hiser et al., 2006]. Furthermore, in bovine brain, the relative protein expression of the different β-tubulin isotypes was 3, 58, 25, 13, 0, and 0% for class I, II, III, IV, V, and VI, respectively [Cleveland et al., 1990], while the human brain mRNA content found in this work for the corresponding genes was 4, 37, 4, 55, 0, and 0%. Concerning cancer cells, a recent work measuring protein levels by quantitative methods in human cell lines from colon, breast, lung, and ovarian tumors found 69, 30, and 2% for class I, IVa/IVb, and III, respectively [Hiser et al., 2006]. This is in agreement with previous reports using different protein quantification techniques [Verdier-Pinard et al., 2005] and also with the mRNA data from this work in colon, breast, lung, and ovarian tumors with an average *TUBB*, *TUBB2C/4*, and *TUBB3* mRNA levels of 51, 37, and 3%, respectively. These data show that *TUBB* is the major isotype in the tumor types tested, in agreement with earlier reports on the relative expression of β-tubulin isotypes in cancer cell lines, xenograft models, and ovarian tumors [Giannakakou et al., 1997; Nicoletti et al., 2001; Ohishi et al., 2007]. Previous studies using lung tissues and breast tumors [Dozier et al., 2003; Cucchiarelli et al., 2008], corroborate the low expression of isotypes IVa and VI, however, the highest mRNA expression in these studies did not correspond to isotype I, but to class V and II for NSCLC and to both class I and V for breast cancer. The discrepancy between the exact percentages identified for each isotype between the study by Nicoletti et al. and our work is likely due to the semi-quantitative nature of the expression data in the earlier report. On the other hand, the discrepancy for class V and II expression with the studies by Cucchiarelli et al. [2008] and Dozier et al. [2003] might be due to differences in the qRT-PCR design, which is shared by both studies (e.g. using gene-specific primers for each isotype in the RT, possible cross-reactions between isotypes or differences in the length of the amplified PCR products).

Concerning the tissue specificity, among the ubiquitous β-tubulin isotypes, *TUBB* and *TUBB2C* were the most abundant isotypes in 19 of the 21 tissues here studied (brain and leukocytes being the exception). In average, together they represented more than 75% of the total β-tubulin content. The contribution of the also ubiquitous isotype *TUBB6* was much smaller, representing in average only 3% of the total β-tubulin content. The amounts of *TUBB2C* exceeded those of *TUBB* in several tissues, its highest expression occurring in testis, as already suggested by studies with homologue proteins [Havercroft and Cleveland, 1984]. *TUBB1* expression was restricted to hematopoietic tissues, while *TUBB2A, TUBB2B, TUBB3*, and *TUBB4* were expressed at very high levels in brain. The large number of brain isotypes likely reflecting the importance of the extensive neuronal cytoskeleton for the diverse functions of the human neurons. When the expression of the so called "neuronal-specific" isotypes was studied in other tissues, it became clear that some of these isotypes were also present at relevant amounts in specific tissues. *TUBB2A* was the isotype with the highest expression outside the brain, followed by *TUBB4, TUBB2B*, and *TUBB3* (see Fig. 2a). Similarly, Arai et al. [2002] showed that brain class II was expressed at low levels in a broad range of tissues.

Interestingly, we noticed that human *TUBB2A* and *TUBB2B* genes are located in tandem in chromosome 6p and encode proteins that only differ in two amino acids. When we examined *Pan troglodytes* genome, *TUBB2A* was not found, suggesting that this gene was recently acquired in humans by duplication of *TUBB2B.* From the two amino acids differentiating the two human class II proteins, at positions 55 Thr/Ala and 201 Cys/Ser, the latter is conserved in all istoypes except for *TUBB2A*, supporting that this is a recent gene. The functional implications of the duplication and the amino acid changes in class IIa protein are unknown, but the similarity between IIa and IIb proteins and the neuronal-specificity suggests a redundancy. On the other hand, the expression of human *TUBB2A* surpassed that of *TUBB2B* in most normal tissues examined, and this difference greatly increased in tumors.

Concerning the β-tubulin expression in tumors, we found that *TUBB* (class I) was the major isotype, followed by *TUBB2C* (class IVb). The antitubulin agents taxol and taxotere exert their antitumoral effect through β-tubulin binding and are clinically used as a first choice treatment for breast, ovary, and lung cancers; however, taxanes are not clinically effective for colon and kidney cancers. Interestingly, *TUBB* was the major isotype in ovary, breast, and lung tumors, but it was also highly expressed in colon and kidney cancers. This suggests that *TUBB* tumoral expression is not the only requirement for taxanes effectiveness, and that other factors such as β-tubulin post-translational modifications or binding of MAPs could also be critical for the drugs' sensitivity [Verhey and Gaertig, 2007; Luchko et al., 2008]. Concerning *TUBB2C*, both mRNA and protein contents were increased in breast cancer cell lines resistant to docetaxel [Shalli et al., 2005] and knockdown of this isotype in vitro increased the sensitivity to *Vinca* alkaloids [Gan and Kavallaris, 2008]. However, overexpression or knockdown of β-tubulin IVb did not seem to influence paclitaxel response in different in vitro models [Blade et al., 1999; Gan and Kavallaris, 2008]. In this work, we found an increased expression of *TUBB2C* in ER

negative compared to ER positive breast tumors (Fig. 4), which could result in differences in drug response. Indeed, several clinical trials have suggested that breast cancer patients with different ER status show differential responses to taxane combined chemotherapy [Berry et al., 2006; Conforti et al., 2007]; however, there are also contradictory results [Andre et al., 2008], which indicate that further research is necessary. The expression of the neuronal-class III has been associated in vitro to altered sensitivity to antitubulin agents [Hari et al., 2003; Kamath et al., 2005] and there is also mounting clinical evidence that it could be involved in tumoral resistance to taxanes [Hasegawa et al., 2003; Mozzetti et al., 2005; Ferrandina et al.,2006, 2007; Seve et al., 2005,2007a,2007b]. In this work, *TUBB3* contribution to the total β-tubulin content in breast, ovary and lung tumors was in average small, but much greater than that in colon and kidney tumors (Figs. 2c and 3d). *TUBB3* reached up to 18 and 16, 7.5% of the total β-tubulin in breast, lung, and ovary cancer samples, respectively, making feasible that, in these cases, *TUBB3* could play role on taxanes sensitivity. Another characteristic of the tumors here studied, was a decreased expression of *TUBB6*, except for kidney (see Figs. 2c, 3e and Supp. Info. Fig. 1e). Class V has been shown in vitro to confer unique properties to the microtubules, disrupting the organization, increasing the fragmentation and resulting in disruption of mitotic spindle assembly and block proliferation [Bhattacharya and Cabral, 2004]. However, normal tissues express various amounts of *TUBB6* (Fig. 2a) and whether *TUBB6* plays an in vivo function or if there is a biological meaning for tumoral *TUBB6* alteration, it remains unknown.

The complex expression patterns of the multiple β-tubulin genes and several in vitro experiments [Banerjee et al., 1990; Panda et al., 1994; Derry et al., 1997] suggest a different functionality for the isotypes; however, there is still no clear in vivo proof supporting this. Alternatively, gene-duplications might have formed multiple genes encoding functionally equivalent proteins that have evolved to possess different regulatory sequences that are activated differentially. Thus, further studies are required to fully understand the biological role of the β-tubulin proteins. This knowledge is crucial to undertake rational studies aimed at the identification of factors influencing tumoral resistance to antitubulin drugs. On the other hand, the differences between the proteins could be directly exploited in the design of antitubulin agents binding to specific β-tubulin isotypes and with lower toxicity. For example, drugs with low affinity for the neuronal specific class IVa (*TUBB4),* which is not expressed in tumors, might retain their tumoral efficacy while showing a lower neurotoxicity, which is a common and serious side effect of the microtubule binding drugs. Furthermore, the recent work by Escuin et al. [2009] has proven that this is feasible clinically, by demonstrating that the low affinity of 2-methoxyestradiol (a microtubule-depolymerizing agent) for the hematopoietic-specific class VI β-tubulin (see Figs. 1b and 2a, 7th and 8th bars) is correlated with the lack of hematologic toxicity in patients receiving 2ME2-based chemotherapy. All together, this work provides key data concerning the normal and tumoral distribution of the most relevant β-tubulin isotypes in different human tissues. These expression patterns could help in the design of novel isotype-specific drugs that could minimize tumoral resistance, while decreasing the toxicities in the patients.

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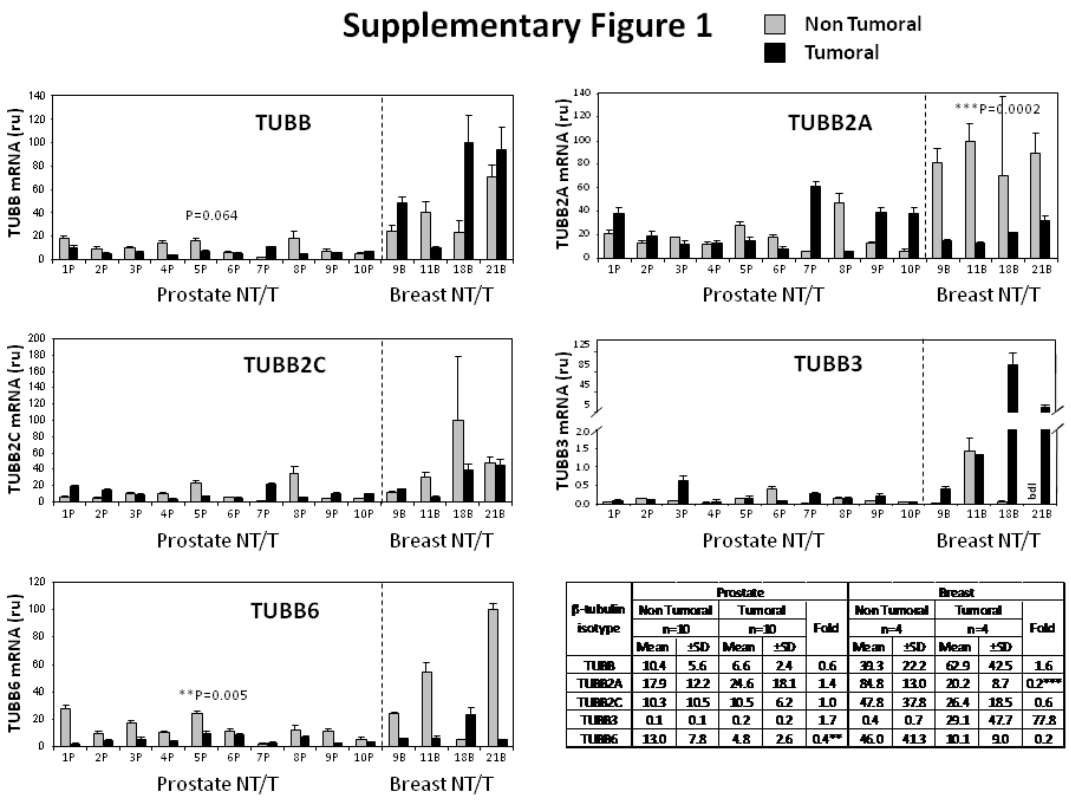
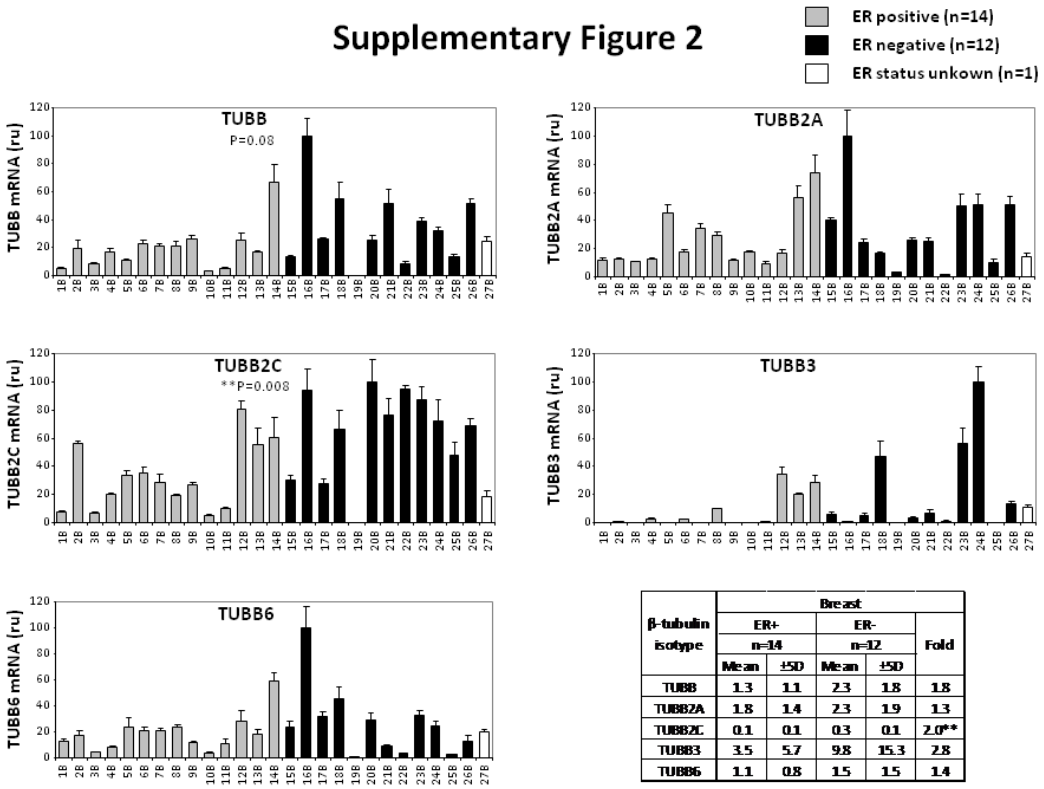
MATERIAL SUPLEMENTARIO

Supplementary Figure 1. Expression of β-tubulin isotypes in human prostate and breast tumors compared to matched non tumoral tissue. The mRNA amounts of the β-tubulin isotypes for 10 prostate and 4 breast matched non-tumoral (NT) and tumoral (T) tissues are shown. The grey bars correspond to non-tumoral and the black bars to tumoral samples. The results are shown for the five isotypes with the highest expression in these samples: **a.** *TUBB*, **b.** *TUBB2A,* **c.** *TUBB2C,* **d.** *TUBB3* and **e.** *TUBB6.* Mean values and standard deviation of each isotype mRNA content as well as the fold change between tumoral and non tumoral samples are summarized in the inserted table.



β-tubulin isotype	Prostate					Breast				
	Non Tumoral n=10		Tumoral n=10		Fold	Non Tumoral n=4		Tumoral n=4		Fold
	Mean	±SD	Mean	±SD		Mean	±SD	Mean	±SD	
TUBB	10.4	5.6	6.6	2.4	0.6	39.3	22.2	62.9	42.5	1.6
TUBB2A	17.9	12.2	24.6	18.1	1.4	84.8	13.0	20.2	8.7	0.2****
TUBB2C	10.3	10.5	10.5	6.2	1.0	47.8	37.8	26.4	18.5	0.6
TUBB3	0.1	0.1	0.2	0.2	1.7	0.4	0.7	29.1	47.7	77.8
TUBB6	13.0	7.8	4.8	2.6	0.4***	46.0	41.3	10.1	9.0	0.2

Supplementary Figure 2. Expression of β-tubulin isotypes in human breast tumors with different estrogen receptor (ER) status. The mRNA amounts of the β-tubulin isotypes in 27 breast tumors are shown. The grey bars correspond to ER positive tumors, the black bars to ER negative tumoral samples and the white bar to ER unknown tumoral sample. The P values comparing the ER positive and negative tumors are shown when significant or near significant values were obtained. The results are shown for the five isotypes with the highest expression in these samples: **a.** *TUBB*, **b.** *TUBB2A,* **c.** *TUBB2C,* **d.** *TUBB3* and **e.** *TUBB6.* The mean values and standard deviation of each isotype mRNA content as well as the fold change between ER positive tumors and ER negative tumors are summarize in the inserted table.



β-tubulin isotype	Breast				
	ER+ n=14		ER- n=12		Fold
	Mean	±SD	Mean	±SD	
TUBB	1.3	1.1	2.3	1.8	1.8
TUBB2A	1.8	1.4	2.3	1.9	1.3
TUBB2C	0.1	0.1	0.3	0.1	2.0**
TUBB3	3.5	5.7	9.8	15.3	2.8
TUBB6	1.1	0.8	1.5	1.5	1.4

ARTÍCULO 2: The miR-200 family controls β-tubulin III expression and is associated with paclitaxel-based treatment response and progression-free survival in ovarian cancer patients.

Autores: Leskelä S*, Leandro-García LJ*, Mendiola M, Barriuso J, Inglada-Pérez L, Muñoz I, Martínez-Delgado B, Redondo A, de Santiago J, Robledo M, Hardisson D, Rodríguez-Antona C.
*** Estos autores han contribuido igualmente.**

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Resumen: El cáncer de ovario sigue siendo una de las principales causas de muerte por cáncer. Existe una urgente necesidad de mejorar las estrategias terapéuticas para este tumor, en parte, mediante la identificación de nuevos marcadores predictivos de respuesta al tratamiento estándar actual con paclitaxel–carboplatino. Los microARNs tienen el potencial de modificar los efectos de los fármacos a través de su capacidad para regular la expresión génica. En este estudio se cuantificó la expresión de los microARNs miR-141, miR-200a, miR-200b, miR-200c y miR-429 y el contenido proteico de los isotipos de β-tubulina I, II y III en una serie de 72 carcinomas de ovario, y demostramos que los tumores con una alta expresión de la familia de microARNs miR-200 mostraban bajos niveles de β-tubulina III (rango de valores de P desde 0.047 a 0.0001). Además, se estudió la relación entre la expresión de los microARNs con la respuesta al tratamiento con paclitaxel-carboplatino, la supervivencia libre de progresión de la enfermedad o PFS (del inglés *Progression Free Survival*) y la supervivencia global o OS (del inglés *Overall Survival*). Aquellos pacientes sin respuesta completa tuvieron una expresión tumoral del miR-200c más baja que los pacientes con respuesta completa, con un riesgo de 1.43, con un intervalo de confianza al 95% de 1.02–1.99 y una P de 0.037 (análisis multivariable). Además, la baja expresión de los miembros de la familia miR-200 se relacionó con una PFS más corta (HR>2.0, P valores de 0.051, 0.054 y 0.079 para el miR-200, el miR-141 y el miR-429, respectivamente; análisis multivariable). En conclusión, los miembros de la familia miR-200 influyen en la expresión proteica de la β-tubulina III en tumores de ovario. Además, en pacientes con carcinoma de ovario avanzado estos microARNs podrían constituir marcadores de respuesta al tratamiento con paclitaxel-carboplatino y de progresión de la enfermedad.

Aportación personal: LJ Leandro-García participó en el diseño del estudio y en el procesamiento de las muestras, retro-transcripción a ADNc y cuantificación de microARNs mediante qRT-PCR. Analizó los datos de la cuantificación y participó en el análisis de la expresión proteica y el análisis estadístico. Finalmente, LJ Leandro-García participó activamente en la discusión crítica de los resultados y fue uno de los dos autores principales en la redacción del trabajo para su publicación.

The miR-200 family controls β-tubulin III expression and is associated with paclitaxel-based treatment response and progression-free survival in ovarian cancer patients

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Abstract

Ovarian cancer remains one of the leading causes of cancer deaths. Thus, new biomarkers predictive of response to the standard paclitaxel–carboplatin treatment are needed to improve chemotherapy strategies. MicroRNAs have the potential to modify drug outcomes. Based on this, we have demonstrated in this study that patients with a high expression of the miR-200 family show low levels of β-tubulin class III in ovarian carcinoma. In addition, we have established the clinical relevance of these microRNAs for ovarian cancer patients' treatment response and survival. In a well-characterized series of 72 ovarian carcinomas, the expressions of miR-141, miR-200a, miR-200b, miR-200c, and miR-429 were quantified by quantitative reverse transcription-PCR, and the protein content of β-tubulin isotypes I, II, and III was determined by immunohistochemistry. The relationship between these microRNAs, β-tubulin expression, response to paclitaxel-based treatment, progression-free survival (PFS) and overall survival was determined. While isotype I had constant high levels, protein expression of β-tubulins II and III was mutually exclusive. Low tumoral miR-200 expression was significantly associated with high β-tubulin III protein content (P values range, 0.047–$<$0.0001), and patients without complete response (CR) had lower miR-200c levels than patients with CR (hazard ratio (HR)$=$1.43, 95% confidence interval (CI)$=$1.02–1.99, $P=0.037$, multivariate analysis). Additionally, low miR-200 family expression had a trend toward poor PFS (HR$>$2.0, P values 0.051, 0.054, and 0.079 for miR-200c, miR-141, and miR-429 respectively, multivariate analysis). In conclusion, miR-200 family members affect the final β-tubulin III protein content of ovarian carcinomas. Furthermore, these microRNAs might constitute the biomarkers of response to paclitaxel-based treatments and relapse/progression of advanced stage ovarian carcinoma patients.

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Introduction

Ovarian cancer is the leading cause of death for gynecologic malignancies (Jemal *et al*. 2009). More than 70% of patients are diagnosed at late stages of the disease and, although the current standard treatment combining surgery with chemotherapy (mainly based on paclitaxel–carboplatin regimens) is efficient in almost 80% of cases, the 5-year survival rate is low due to the high incidence of recurrence and to the ultimate resistance to taxanes and/or platinum-based drugs (Ozols *et al*. 2003, Heintz *et al*. 2006). New biomarkers predictive of treatment response urgently need to be identified in order to improve chemotherapy strategies.

First-line treatment for advanced ovarian cancer consists of a combination of paclitaxel and carboplatin (Ozols *et al*. 2003, Omura 2008). Carboplatin is equally efficient but less toxic and easier to administer than cisplatin, thus, it replaced cisplatin as the standard treatment for ovarian cancer (Ozols *et al*. 2003, Omura 2008). Paclitaxel is an antimitotic drug, which alters the dynamics of the cellular microtubules, which maintain cellular structure and are essential for diverse cellular functions such as cell cycle, cell signaling, and intracellular trafficking (Seve & Dumontet 2008). Paclitaxel binds to β-tubulin, leading to cellular microtubules stabilization, mitotic arrest, and finally to cell death. In humans, there are at least eight different isotypes of β-tubulin, which exhibit an altered expression pattern in tumoral tissue (Leandro-Garcia *et al*. 2010). In ovarian carcinoma, high protein levels of classes I and IV, intermediate levels of class III, and low levels of class II β-tubulin have been reported (Ohishi *et al*. 2007). High tumoral β-tubulin III expression has been associated with worse survival in non-small cell lung cancer (Rosell *et al*. 2003, Seve *et al*. 2005), breast (Seve & Dumontet 2008), head and neck (Koh *et al*. 2009), and ovarian cancer (Ferrandina *et al*. 2006), although Aoki *et al*. (2009) reported a better survival for patients with ovarian clear cell adenocarcinoma positive for class III expression. In ovarian cancer, β-tubulin III has also been associated with worse treatment response (Kavallaris *et al*. 1997, Mozzetti *et al*. 2005, Umezu *et al*. 2008). On the other hand, absence of class II β-tubulin expression has been associated with advanced stage and short progression-free survival (PFS) in ovarian tumors (Ohishi *et al*. 2007).

The molecular mechanisms leading to the upregulation of class III β-tubulin in tumors remain largely unknown. Hypoxia-inducible factor 1 seems to play a role in ovarian carcinomas (Raspaglio *et al*. 2008), and epigenetic modifications have been suggested to be a contributing factor in ovarian tumors (Izutsu *et al*. 2008) and melanoma cells (Akasaka *et al*. 2009). Recent studies suggest an important role of microRNAs, specifically, Cochrane *et al*. (2009, 2010) demonstrated that miR-200c had a binding site in the 3′ untranslated region (UTR) of class III β-tubulin and that overexpression of miR-200c in cell lines decreased β-tubulin III protein content and restored sensitivity to microtubule-targeting agents. Interestingly, the miR-200 family, formed by five microRNAs (miR-141, miR-200a, miR-200b, miR-200c, and miR-429) and located in two clusters in the genome, is involved in the epithelial to mesenchymal transition (EMT) through regulation of E-cadherin expression via suppression of ZEB1 and ZEB2 (Gregory *et al*. 2008, Korpal *et al*. 2008, Park *et al*. 2008). In ovarian cancer, a recent study with 55 advanced tumor samples showed that a high expression of miR-200a, miR-200b, and miR-429 was associated with improved survival (Hu *et al*. 2009). Nevertheless, another study including 20 serous carcinomas found that high expression of the miR-200 family members significantly correlated with poor prognosis (Nam *et al*. 2008). Thus, although there is evidence suggesting that the miR-200 family might play a key role in the response to microtubule-binding drugs and ovarian cancer survival, the relevance of these microRNAs as clinical markers for patients is largely unknown.

In this study, we quantified the expression of the miR-200 family in a well-characterized series of 72 epithelial ovarian tumors and examined their contribution to the protein expression of β-tubulin isotypes I, II, and III. We also investigated the impact of these microRNAs on the patients' response to and survival following paclitaxel-based therapy. The data provided in this study improved our comprehension of treatment failures in ovarian carcinoma and indicated the miR-200 family as a potential novel target for improved treatment strategies.

Materials and methods

Patient selection

This study included 72 formalin-fixed and paraffin-embedded ovarian carcinoma samples from patients treated at the Hospital Universitario La Paz (HULP), Madrid, Spain. All patients underwent a baseline computed tomography (CT) scan and exploratory laparotomy for diagnosis, staging, and debulking when feasible. All patients received a platinum/taxane-based chemotherapy for at least six cycles. Patients were divided into stages according to the International

Federation of Gynecology and Obstetrics (FIGO) classification. Optimal debulking was defined as ≤ 1 cm (diameter) residual disease. A complete response (CR) was defined as absence of all clinical/radiographic evidence of disease. In addition, a second-look laparotomy (SLL) was performed on most of the patients who achieved a CR after planned treatment and all of them who were optimally debulked. In patients who achieved a CR after the planned treatment and did not accept an SLL or for whom this procedure was not feasible, and in patients with a partial response (PR), a second CT scan was performed 1 month after the first evaluation to confirm the response. Follow-up data were obtained by retrospective chart review. PFS was defined as the time interval between the start of the treatment and the first confirmed sign of disease recurrence or progression. Overall survival (OS) was defined as the time interval between the start of the treatment and the date of death or end of follow-up. Approval for the study was obtained from the local ethics committee. Relevant clinicopathological data of the patients are shown in Table 1.

Treatment response was studied in a homogeneous subgroup of 57 patients with both advanced tumor stage (FIGO stages III and IV) and serous carcinoma histology. In this subgroup of patients, 84 and 16% of the tumors corresponded to III and IV stages respectively. Regarding tumor grade in this subset of tumors, 61, 32, and 7% of the tumors were grades 3, 2, and 1 respectively. The clinical response to the treatment, debulking status after surgery, and survival data in this subgroup of patients are presented in Table 2.

Table 1 Clinicopathological data of the ovarian cancer patients[a]

	All cases ($n=72$)		Serous III/IV ($n=57$)[b]	
	n	**%**	***n***	**%**
Age (years)				
Median	57.0		54.0	
(minimum–maximum)	(35–85)		(35–85)	
Histological subtype				
Serous carcinoma	57	80	57	100
Clear cell carcinoma	6	8	–	–
Endometrioid carcinoma	4	6	–	–
Mucinous carcinoma	3	4	–	–
Mixed endometrioid–clear cell carcinoma	1	1	–	–
Mixed endometrioid–serous carcinoma	1	1	–	–
FIGO stage				
I	3	4	–	–
II	5	7	–	–
III	54	75	48	84
IV	10	14	9	16
Tumor grade				
Well differentiated (grade 1)	8	11	4	7
Moderately differentiated (grade 2)	24	33	18	32
Poorly differentiated (grade 3)	39	54	35	61
Unknown	1	1	–	–

FIGO, International Federation of Gynecology and Obstetrics.
[a]All patients included in the study were Caucasian females.
[b]Clinicopathologial characteristics of the subset of 57 patients with FIGO stages III and IV and serous ovarian carcinoma.

Tissue microarray construction

Representative areas of the tumors were selected on hematoxylin and eosin-stained sections and marked on individual paraffin blocks. Two tissue cores (1 mm in diameter) were obtained from each specimen. The tissue cores were arrayed into a receptor paraffin block using a tissue microarray workstation (Beecher Instruments, Silver Spring, MD, USA) as previously described (Hardisson *et al*. 2003). A hematoxylin and eosin-stained section of the array was reviewed to confirm the presence of morphologically representative areas of the original lesions.

Immunohistochemistry

Immunohistochemistry was performed on 4 μm sections of formalin-fixed, paraffin-embedded tissues. Briefly, the tissue sections were deparaffinized and rehydrated in water, after which antigen retrieval was carried out by incubation in EDTA solution, pH 8.2 at 50 °C for 45 min in an autoclave. Endogenous peroxidase and nonspecific antibody reactivity were blocked with peroxidase-blocking reagent (Dako, Glostrup, Denmark) at room temperature for 15 min. The sections were then incubated for 60–90 min at 4 °C with the following antibodies: class I β-tubulin (clone SAP.4G5, Sigma–Aldrich, dilution 1:100), class II β-tubulin that recognizes classes IIa and IIb β-tubulins, which differ in one single amino acid, (clone 7B9, Covance, Emeryville, CA, USA, dilution 1:100), and class III β-tubulin (clone TUJ-1, Santa Cruz Biotechnology, Heidelberg, Germany, dilution 1:200). Detection was performed with Envision Plus Detection System (Dako).

Immunohistochemical results were evaluated and scored by one pathologist (D H) blinded to the clinical data of the patients. Immunoreactivity was scored by estimating the percentage of tumor cells with cytoplasmic immunostaining, regardless of intensity. The intensity of the immunostaining was not considered

Table 2 Clinical characteristics of patients with International Federation of Gynecology and Obstetrics (FIGO) stages III and IV and serous ovarian carcinoma

	n	%
Response to treatment		
Complete response	38	67
Partial response	11	19
Stable disease	3	5
Progressive disease	4	7
Unknown	1	2
Pathological response		
Complete response	14	25
Stable disease	10	17
Unknown	33	58
Debulking status		
Optimal (<1 cm)	24	42
Suboptimal (>1 cm)	12	21
Unknown	21	37
Relapse/progression		
Yes	47	83
No	8	14
Unknown	2	3
Progression-free survival (months)		
Median	16.0	
(minimum–maximum)	(1–128)	
Deceased		
Yes	35	61
No	19	33
Unknown	3	5
Overall survival (months)		
Median	35.1	
(minimum–maximum)	(1–128)	

since in our study the intensity of the immunostaining in the positive tumors was predominantly high, precluding discrimination between the samples. Class I β-tubulin expression was not categorized since the expression levels of all samples were consistently high. In the tissue sections, negative controls were used with only the secondary, replacing the primary antibody.

RNA isolation and real-time quantitative reverse transcription-PCR

Tissue sections previously stained with hematoxylin and eosin were reviewed by an experienced pathologist. Eligible samples included at least 80% of tumor cells and no large necrotic areas. Four to eight 4 μm sections were used for RNA isolation with the Masterpure RNA Purification kit (EPICENTRE Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions. For microRNA quantitative reverse transcription (qRT)-PCR, 25 ng total RNA were reverse transcribed using the miRCURY LNA First-Strand cDNA kit (Exiqon A/S, Vedbaek, Denmark) and the miRCURY LNA microRNA Primer Sets (Exiqon) corresponding to hsa-miR-141, hsa-miR-200a, hsa-miR-200b, hsa-miR-200c, hsa-miR-429, and the control primer set 5S rRNA, according to the manufacturer's instructions. Negative controls with reaction mix without reverse transcriptase were included for the different microRNAs studied. Real-time qPCR was performed with the Sequence Detection System 7900HT (Applied Biosystems, Carlsbad, CA, USA) using the miRCURY LNA SYBR Green Master Mix (Exiqon), following the manufacturer's instructions. The amplification conditions consisted of an initial step at 95 °C for 10 min, followed by 50 cycles of 20 s at 95 °C and 1 min at 60 °C. Negative controls were included in all PCRs, and all assays were performed in triplicate. The $\Delta\Delta C_t$ method was used for the calculation of the different amounts of mRNA (Livak & Schmittgen 2001). Normalization was carried out with the endogenous control 5S ribosomic RNA. The relative abundance of different miR-200 family members cannot be precisely estimated because the hybridization characteristics of the different qRT-PCR probes could vary, however, approximate conclusions can be drawn due to the large differences found (Supplementary Figure 3, see section on supplementary data given at the end of this article).

Statistical analysis

All statistical analyses were carried out using SPSS version 17.0 statistical software (SPSS Inc., Chicago, IL, USA). The association between the expression levels of the five members of the miR-200 family and continuous demographic variables (such as age) was determined by the Pearson coefficient. β-Tubulins II and III protein expression, treatment response, pathological response, mortality, relapse, and tumoral characteristics (histology subtype, differentiation grade, and tumor stage) were analyzed as categorical variables. Similar to previous publications, protein expression of β-tubulins II and III was used as a binary variable (low expression versus high expression) using 75% of positive cells as the cutoff (Ohishi *et al*. 2007). The response to treatment was divided into two categories: patients with CR and those with PR, stable disease, and disease progression grouped together. Associations between the response to treatment and clinical variables (histology subtype, differentiation grade, tumor stage, and patient's age) were evaluated with χ^2 test, Fisher's exact test, and Student's *t*-test, when appropriate. P values <0.05 were considered statistically significant and all of them are two-sided. To analyze the associations between miR-200 expression and β-tubulin content and treatment

response, since the tumoral miR-200 family content followed a normal distribution (Kolmogorov–Smirnov test), Student's *t*-tests were used, applying the Welch correction when the S.D.s differed significantly between the groups. To further analyze miR-200 expression and response to treatment, logistic regression was applied and the hazard ratio (HR) was estimated, adjusting for relevant clinicopathological variables. To analyze miR-200 expression and PFS and OS, the univariate analysis was carried out by using the Kaplan–Meier plots coupled to log-rank test and univariate Cox regression model was applied for the HR estimation. The multivariate Cox proportional hazards regression model was used to evaluate the prognostic significance of the microRNA adjusted by clinicopathological variables.

Results

Predicted binding sites of miR-200b/200c/429 in the β-tubulin 3′ UTR and immunohistochemical determination of β-tubulin isotypes I, IIa, and III expression

Recently, *in vitro* studies have shown that the 3′ UTR region of class III β-tubulin has a miR-200c-binding site. The important role that this microRNA plays in metastasis and the high degree of conservation among β-tubulin isotype functions and genetic structure led us to further explore in tumor samples the correlation between the expression of the tubulin isoforms and the miR-200 family. By means of an *in silico* analysis (http://www.targetscan.org/), miR-200b/200c/429-binding sites were predicted in the 3′ UTR not only of class III β-tubulin but also of classes I and IIa, while no binding sites were predicted in the rest of the human β-tubulin isotypes. The miR-200b/200c/429 microRNA-binding site was broadly conserved among vertebrates for classes I and III β-tubulins, but poorly conserved for class IIa. The *in silico* analysis did not predict miR-141 and miR-200a binding, due to one nucleotide difference in the seed sequence (Supplementary Figure 1, see section on supplementary data given at the end of this article), but there is evidence for miR-200 family common targets.

We then determined the protein expression of classes I, II, and III β-tubulins in 72 ovarian cancer samples by immunohistochemical analysis (Fig. 1). The protein staining showed substantial differences for the different isotypes, while class I protein expression showed no variation among the cases, β-tubulins II and III exhibited substantial intersample differences. All samples exhibited a very strong staining for class I β-tubulin; class II protein was absent in 46 (69%) tumors, while 15 cases had low and 6 cases had a high protein expression (22 and 9% of the tumors respectively). Class III protein was absent in 34 (48%) cases, while 29 (41%) had low and 8 (11%) had a high protein expression (Supplementary Figure 2A, see section on supplementary data given at the end of this article). With regards to the correlation between the different isotypes, classes II and III β-tubulin expression proved to be mutually exclusive events, with samples exhibiting a high β-tubulin class III content lacking isotype II expression and vice versa (Supplementary Figure 2B, see section on supplementary data given at the end of this article).

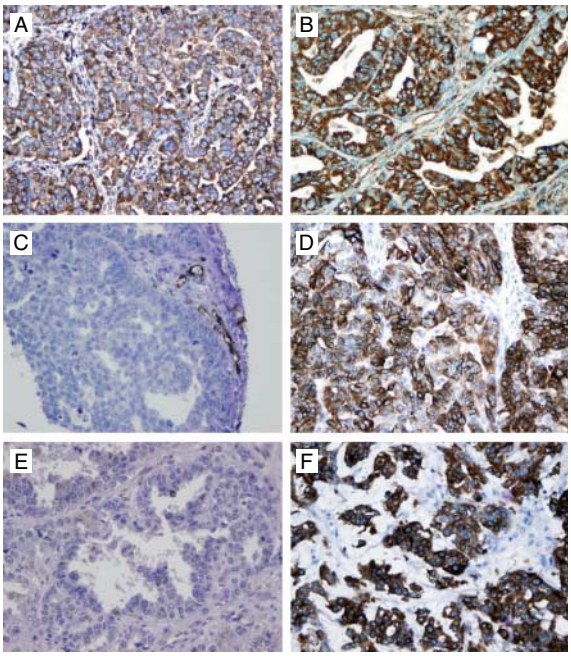

Figure 1 Protein expression of β-tubulin isotypes I, II, and III in ovarian carcinomas. Isotype I exhibited a high expression in all cases analyzed with minimal differences among samples (A) and (B). Illustrative cases with low (C) and high (D) β-tubulin isotype II expression. Illustrative cases with low (E) and high (F) β-tubulin isotype III expression. All cases shown correspond to serous carcinomas.

Expression of the miR-200 family is associated with tumoral β-tubulin III protein expression

To investigate whether the miR-200 family could regulate β-tubulin isotypes I, II, and III, we measured the expression of these microRNAs in ovarian cancer samples. The expression levels found were variable, with miR-200c expressed at the highest level, then, miR-200b, miR-200a, and finally miR-429 and miR-141, which were expressed at similarly low-expression levels (Supplementary Figure 3, see section

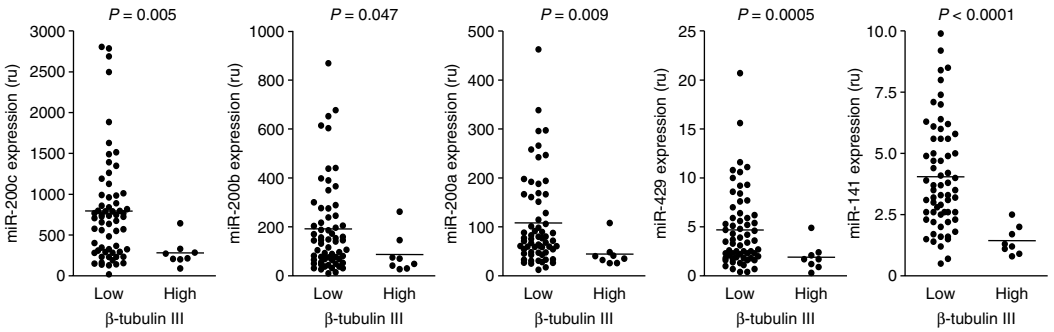

Figure 2 Tumors with high levels of β-tubulin III protein have significantly decreased miR-200 expression. Samples with high isotype III expression (more than 75% positive cells) showed a significantly lower miR-200c, $P=0.005$; miR-200b, $P=0.047$; miR-200a, $P=0.009$; miR-429, $P=0.0005$; and miR-141, $P<0.0001$ expression compared with samples with low isotype III expression. MicroRNAs are shown in the figure according to the expression levels. To express the microRNAs content as whole numbers, their expression was multiplied by 100 and expressed as relative units (ru).

on supplementary data given at the end of this article). As expected, correlations were found among the miR-200 family members, with miR-141/miR-200a and miR-200a/miR-200b showing the highest correlation and miR-429 the lowest (Supplementary Table 1, see section on supplementary data given at the end of this article).

We then determined whether the miR-200 family could regulate the protein expression of β-tubulin isotypes I, II, and III. We found a statistically significant association between class III β-tubulin protein expression and the tumoral content of all miR-200 members (Fig. 2). According to this, the ovarian tumors with low miR-200 expression exhibited high levels of class III protein, suggesting that the absence of these microRNAs in the tumor results in lack of class III β-tubulin degradation and accumulation of high levels of the protein. The strongest associations corresponded to miR-141, miR-429, and miR-200c ($P<0.006$) among which miR-200c showed the highest expression. No association was found between protein levels of β-tubulins I and II with miR-200 family expression (data not shown).

miR-200c expression determines the response to paclitaxel-based chemotherapy in serous ovarian carcinoma patients

Owing to the importance of tumor type and stage, we selected a homogenous subgroup of patients with serous carcinomas and advanced tumor stage (FIGO stages III and IV) to study whether the miR-200 family could influence clinical response to treatment: fifty-seven patients met these inclusion criteria. In these samples, we did not find statistically significant associations between the expression of classes I, II, or III β-tubulin content and response to treatment, relapse, or survival of the patients. Notwithstanding this, we found a statistically significant association between miR-200c expression and response to treatment ($P=0.0027$ with t-test; HR = 1.43, 95% CI = 1.02–1.99, $P=0.037$ with logistic regression multivariate analysis, Table 3). The patients who did not achieve a complete clinical response had lower miR-200c levels than those patients with CRs. A significant association was also found between miR-200c and pathological response using the t-test ($P=0.045$), although it did not reach significance in a logistic regression multivariate analysis (HR = 1.45, 95% CI = 0.94–2.25, $P=0.094$). With respect to the number of recurrence and mortality events, low expression of miR-200c was associated with recurrence (odds ratio (OR) = 1.17, 95% CI = 1.01–1.34, $P=0.030$), while no significant association was observed for mortality (OR = 1.11, 95% CI = 0.97–1.28, $P=0.128$; Table 3). miR-200c expression did not show any association with other clinicopathological characteristics. No association with treatment response was found for any of the other miR-200 family members.

miR-200 family expression is associated with prognosis in serous ovarian carcinoma patients

Of the miR-200 family, only miR-429 expression showed a statistically significant association with the recurrence-free survival and OS of the patients (HR = 2.01, 95% CI = 1.11–3.66, $P=0.021$ and HR = 2.08, 95% CI = 1.03–4.20, $P=0.041$; Fig. 3). The PFS rate at 12 months post treatment was 85% in the group of high miR-429 expression and 48% for those with low expression. For miR-200c, the PFS rate at 12 months was 73 and 54% for high and low expressors respectively. After the multivariable analysis, adjusting for relevant clinicopathological

Table 3 miR-200c expression and response to paclitaxel–carboplatin chemotherapy

	Number of samples	miR-200c		Difference of means	Logistic regression	
		Mean	95% CI (ru)	*P* value[a]	HR (95% CI)	*P* value[b]
Response						
CR	37	9.1	(6.8–11.3)	**0.0027**[c]	**1.43 (1.02–1.99)**	**0.037**
No CR (PR, SD, PD)	17	4.8	(3.1–6.5)			
Pathological response						
CR	14	8.0	(5.6–10.3)	**0.045**	1.45 (0.94–2.25)	0.094
No CR	10	4.6	(2.0–7.1)			
Recurrence						
No	8	11.8	(3.1–20.6)	0.243[c]	**1.17 (1.01–1.34)**	**0.030**
Yes	45	7.0	(5.5–8.5)			
Deceased						
No	19	9.3	(5.4–13.1)	0.249[c]	1.11 (0.97–1.28)	0.128
Yes	35	6.9	(5.0–8.7)			

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease. Values in bold are statistically significant.
[a]Univariate analysis using unpaired *t*-test.
[b]Multivariate analysis using logistic regression with debulking status, tumor grade, and FIGO stage as covariates.
[c]The Welch correction was applied when the S.D. differed significantly between the groups.

variables (debulking status, tumor stage, and histological grade), a tendency was observed for miR-429 expression to associate with recurrence-free survival (HR = 2.10, 95% CI = 0.92–4.79, $P = 0.079$). Similarly, miR-200c and miR-141 also showed this trend (HR = 2.24, 95% CI = 1.00–5.03, $P = 0.051$ and HR = 2.35, 95% CI = 0.98–5.59, $P = 0.054$ respectively) (Supplementary Table 2, see section on supplementary data given at the end of this article). When analyzing OS in the multivariate analysis, the association was not statistically significant for miR-429 or the other microRNAs.

Discussion

Ovarian cancer remains one of the leading causes of cancer death. Most ovarian cancers are detected at advanced stages and, although substantial progress has been made in the treatment of this tumor, lack of response and relapse due to intrinsic or acquired resistance greatly reduce survival rates. Thus, there is a need to improve patient care through the identification of biomarkers predictive of treatment response. This study focuses on the new field of microRNAs, because of their potential to provide novel drug response markers (Yang *et al*. 2008*a*,*b*, Adam *et al*. 2009, Li *et al*. 2009), and gives insight into the role of the miR-200 family in paclitaxel–carboplatin response and survival.

The miR-200 family has been shown to maintain the cellular epithelial phenotype via repression of ZEB1 and ZEB2 and to play an important role in tumor progression (Gregory *et al*. 2008). Interestingly, using *in silico* tools we have found that miR-200b/200c/429 had putative binding sites in the 3′ UTR of the β-tubulin isotypes I, IIa, and III (Supplementary Figure 1, see section on supplementary data given at the end of this article). Since β-tubulin is the therapeutic target of paclitaxel, we speculated that these microRNAs might influence the response of ovarian cancer to paclitaxel-based treatments through the downregulation of these isotypes in the tumoral cells. The miR-200 family of microRNAs has seed sequences differing by one nucleotide (Supplementary Figure 1, see section on supplementary data given at the end of this article) and, although target prediction algorithms assume significant differences in the genes targeted by miR-200b/200c/429 and miR-200a/141, there is evidence indicating a high degree of overlap in target genes (Park *et al*. 2008). This data suggests that multiple members of the miR-200 family may target a large common subset of genes to enhance the efficiency of genetic regulation.

When we measured the expression of the five miR-200 family members in 72 epithelial ovarian cancer samples (Supplementary Figure 3, see section on supplementary data given at the end of this article), we confirmed a high degree of variation (Park *et al*. 2008, Hu *et al*. 2009). The protein expression of classes I, II, and III β-tubulin (Supplementary Figure 2, see section on supplementary data given at the end of this article) showed isotype-specific differences, similar to those reported in a previous study (Ohishi *et al*. 2007). Interestingly, we found classes II and III β-tubulin

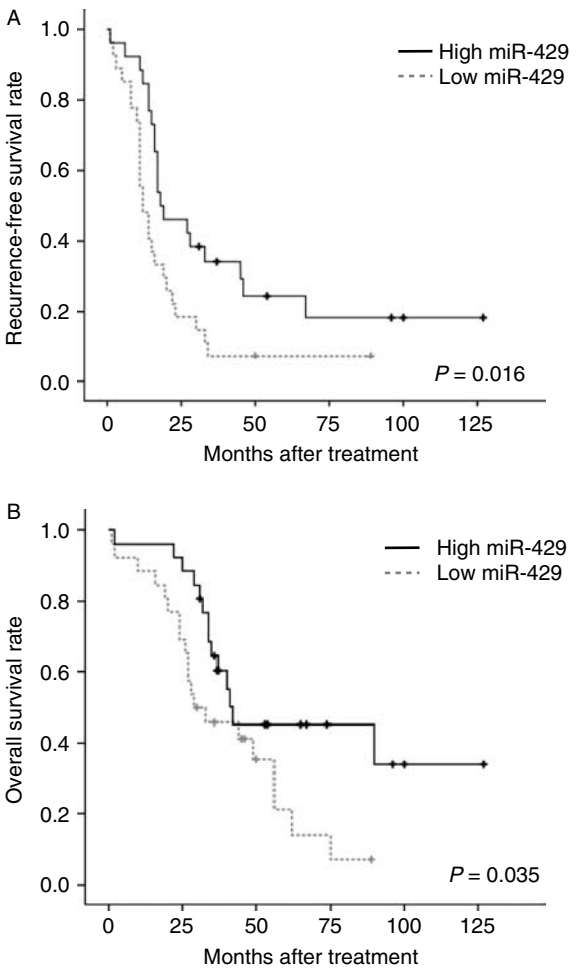

Figure 3 miR-429 is associated with recurrence-free survival and overall survival. The Kaplan–Meier survival analysis evaluating the effect of miR-429 expression on disease outcome for (A) recurrence-free survival and (B) overall survival. The miR-429 median expression was used as cutoff value to divide the patients into low and high miR-429 expressors. The median progression-free survival was 12 months (95% CI 8.2–15.8) for the low and 18 months (95% CI 8.8–27.2) for the high miR-429 expressors. *P* values shown correspond to the log-rank test.

expression to be mutually exclusive, suggesting a complex regulatory mechanism. In other tumor types, isotype III has been detected in 36% of gastric (Urano *et al*. 2006), 40% of head and neck (Koh *et al*. 2009), and 84% of breast (Paradiso *et al*. 2005) cancer samples respectively. β-tubulin III tumoral overexpression has been associated with poor prognosis in a variety of cancer types (Seve *et al*. 2005, Seve & Dumontet 2008, Koh *et al*. 2009), including ovarian carcinomas (Ferrandina *et al*. 2006), although there is one contradictory report (Aoki *et al*. 2009) in clear cell ovarian cancer patients. Concerning response to taxanes, which is closely related to survival rates, high β-tubulin III protein expression was reported to be associated with lack of response in breast (Hasegawa *et al*. 2003, Paradiso *et al*. 2005), lung (Rosell *et al*. 2003, Seve *et al*. 2005), and ovarian (Umezu *et al*. 2008) cancers. In our study, we were not able to detect a significant association between β-tubulin III expression and treatment response, probably due to a small number of high level class III samples. Altogether, these findings seem to reflect an increased resistance of class III to the effect of microtubule-binding drugs (Cochrane *et al*. 2009). In contrast, lack of isotype II expression has been associated with advanced stage and short PFS in ovarian cancer (Ohishi *et al*. 2007). These findings are in agreement with the mutual exclusivity we found for β-tubulins II and III expression.

Interestingly, we found that low levels of miR-200 were associated with high levels of class III protein, implying that β-tubulin III expression could be regulated by this family of microRNAs in clinical samples. In support of this finding, it has been recently shown that the reinstatement of miR-200c in cell lines decreases class III β-tubulin expression and increases sensitivity to microtubule-targeting agents (Cochrane *et al*. 2009) through direct targeting of β-tubulin III (Cochrane *et al*. 2010). However, it should be noted that samples with low levels of miR-200 did not always exhibit high levels of β-tubulin III (Fig. 2). This suggests that in addition to microRNA depletion, other mechanisms, such as epigenetic modifications, are required for β-tubulin III upregulation. We did not find an association between miR-200 and β-tubulins I and II, suggesting that the predicted binding sites for miR-200 in β-tubulins I and IIa genes are either nonfunctional or alternative mechanisms are crucial for the regulation of the eventual protein expression. Mutations on the miRNA-binding sites and alternative cleavage or polyadenylation of the 3′ UTRs are frequent in cancer and might be the mechanism underlying this observation (Blenkiron & Miska 2007, Mayr & Bartel 2009).

Additionally, for class IIa the antibody available for immunohistochemistry detected both IIa and IIb isotypes, which differ in a single nucleotide, and the predicted binding site in IIa was only conserved among mammals. However, for class I the predicted binding site was broadly conserved among vertebrates, similar to that of isotype III.

We then explored a possible role for miR-200 expression as a marker of response to paclitaxel–carboplatin regimen in ovarian carcinomas. Owing to the impact of the cancer stage and histology on response to treatment, we analyzed a homogenous

series of serous adenocarcinomas with FIGO stages III and IV. We found a significant association between miR-200c expression and treatment response: women lacking CR had tumors with significantly lower miR-200c levels than the ones who had achieved CR (HR = 1.43, 95% CI = 1.02–1.99, $P = 0.037$; Table 3); in addition, higher expression of miR-200c was associated with lower relapse/progression rates (HR = 1.17, 95% CI = 1.01–1.34, $P = 0.030$; Table 3). These data seem to indicate that a low miR-200c expression results in high β-tubulin III expression and, thus, increased resistance to paclitaxel-based therapies. *In vitro* studies further support this connection (Cochrane *et al*. 2009, 2010). Regarding prognosis, we found that low tumoral miR-429 was associated with poor PFS and OS (Fig. 3). Multivariate analysis adjusted to relevant clinicopathologic variables revealed a trend for miR-429, miR-200c, and miR-141 with PFS (Supplementary Table 2, see section on supplementary data given at the end of this article), while the association with OS was lost. Hu *et al*. (2009) found a statistically significant association of miR-200a expression with OS and PFS of cancer patients, but Nam *et al*. (2008) described opposite results for the miR-200 family. The discrepancy found by Nam *et al*. could be caused by the small number of samples included in the study (20 serous ovarian carcinoma samples).

Since low tumoral expression of the miR-200 family has been associated with tumor progression and metastasis (Gregory *et al*. 2008, Park *et al*. 2008, Baffa *et al*. 2009), this could lead to a lower OS, independent of treatment response. Our results suggest a possible role for the miR-200 family members as predictive factors for paclitaxel-based response, especially miR-200c, and as prognostic factors in ovarian carcinoma. Because all miR-200 family members share similar targets, but there are differences in the recognition site, we propose that specific members of the family might be more important for prognosis and others for treatment response. In addition, the relative expression levels in the tumor cells could be playing a role in the final regulation of target genes. Thus, the effect of low tumoral miR-200 family expression could be twofold: a decreased response to microtubule-binding drugs and an increased metastasis risk through increased EMT.

Whether the relationship between the expression of miR-200 and first-line treatment response is due to paclitaxel alone or due to combined therapy is unknown, however, this could only be studied using single-agent paclitaxel. Even so, in the context of the new regimens with better response rates (such as combination with antiangiogenic compounds (Burger *et al*. 2007, Cannistra *et al*. 2007, Penson *et al*. 2010)), markers which are able to identify patients who efficiently respond to carboplatin/paclitaxel treatment are relevant. This subset of patients might not benefit from newly targeted drugs, especially if their risk of serious adverse reactions (e.g. bowel perforation) is increased.

In conclusion, we suggest that miR-200 down-regulates β-tubulin III in ovarian tumors. Furthermore, our results suggest a possible role for the miR-200 family both as a prognostic factor and a marker of treatment failure in ovarian carcinoma. Thus, miR-200 might constitute an important biomarker for ovarian cancer patients and could provide the basis for future therapies restoring miR-200 expression in tumor cells. Nevertheless, these data should be further validated in independent cohorts and prospective trials.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1677/ERC-10-0148.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

C Rodríguez-Antona, D Hardisson, M Robledo, and B Martínez-Delgado contributed to the study design; D Hardisson, M Mendiola, J Barriuso, A Redondo, and J de Santiago conceived data collection; S Leskelä, L J Leandro-García, M Mendiola, I Muñoz, and J Barriuso carried out the experiments; S Leskelä, L J Leandro-García, L Inglada, J Barriuso, and C Rodríguez-Antona analyzed the data. All authors were involved in writing the article and had final approval of the submitted and published versions.

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MATERIAL SUPLEMENTARIO

Supplementary table 1. Correlation between the expression of the miR-200 family members in 72 ovarian carcinomas. Pearsson correlation coefficients are shown together with P values in brackets. Statistically significant P values are shaded in light grey ($P<0.05$), extremely significant values in dark grey ($P<10^{-6}$).

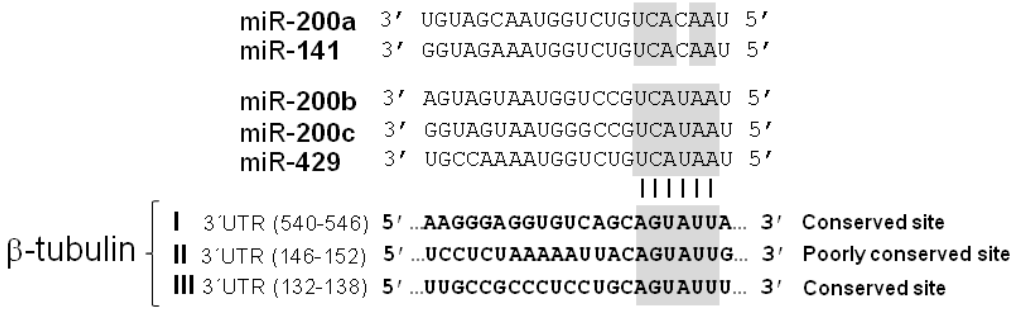
	miR-141	**miR-200a**	**miR-200b**	**miR-200c**	**miR-429**
miR-141		0.554** (0.0000004)	0.400** (0.0005)	0.441** (0.0001)	0.232 (0.050)
miR-200a	0.554** (0.0000004)		0.603** (0.00000002)	0.124 (0.30)	0.352** (0.002)
miR-200b	0.400** (0.0005)	0.603** (0.00000002)		0.279* (0.018)	0.180 (0.13)
miR-200c	0.441** (0.0001)	0.124 (0.30)	0.279* (0.018)		0.124 (0.30)
miR-429	0.232 (0.050)	0.352** (0.002)	0.180 (0.13)	0.124 (0.30)	

Supplementary table 2. miR-200 family expression and progression-free survival.

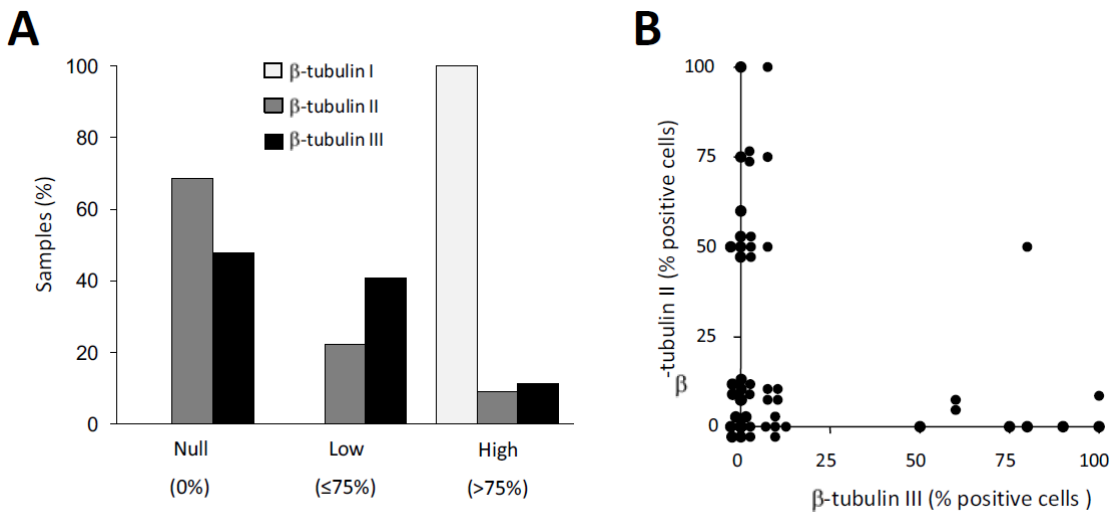
microRNA	HR (95%CI)[a]	*P* value[a]
miR-200c	2.24 (1.00-5.03)	0.051
mirR-200b	1.35 (0.62-2.93)	0.045
miR-200a	1.22 (0.57-2.58)	0.612
miR-141	2.35 (0.98-5.59)	0.054
miR-429	2.10 (0.92-4.79)	0.079

[a]Hazard ratios (HR) and P values for progression-free survival were calculated using multivariate Cox regression analysis with debulking status, tumor grade and FIGO stage as covariates.

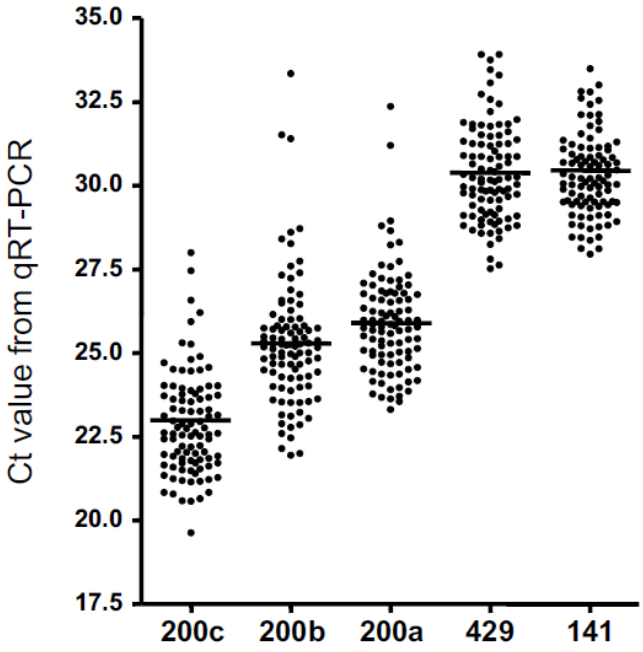
Supplementary Figure 1. Predicted miR-200b/200c/429 binding sites in the 3'UTR of β-tubulin isotypes I, II and III. The six base pairs seed region common for miR-200b/200c/429 is shown together with the predicted binding sites in the 3'UTR regions of β-tubulin isotypes I, II and III. The miR-200a and miR-141 recognition sequence is also shown including the nucleotide change in the seed sequence. The location, with respect to the stop codon, of the miR-200 binding sites in the 3'UTR of the β-tubulin isotypes is shown in brackets.



Supplementary Figure 2. Distribution of ovarian carcinomas according to beta-tubulin isotypes I, II and III protein expression. (A) All the carcinomas showed a strong isotype I staining, while isotypes II and III exhibited substantial differences in protein levels amongst the carcinomas. Null, indicates lack of staining, Low, indicates that less than or equal to 75% of the tumor cells showed positive immunostaining of the corresponding protein, and High, indicates that >75% of cells exhibited positive staining. β-tubulin isotypes I, II and III correspond to light grey, dark grey and black bars, respectively. (B) Mutually exclusive protein expression of β-tubulin isotypes class II and III.



Supplementary figure 3. miR-200 family expression in ovarian tumors. The expression of miR-141, miR-200a, miR-200b, miR-200c and miR-429 was measured by qRT-PCR, as described in Materials and Methods section in 72 ovarian tumors. The Ct-values of each microRNA are shown in the figure.



ARTÍCULO 3: Regulatory polymorphisms in β-tubulin IIa are associated with paclitaxel-induced peripheral neuropathy

Autores: Luis J. Leandro-García, Susanna Leskelä, Carlos Jara, Henrik Gréen, Elisabeth Åvall-Lundqvist, Heather E. Wheeler, M. Eileen Dolan, Lucia Inglada-Perez, Agnieszka Maliszewska, Aguirre A de Cubas, Iñaki Comino-Méndez, Veronika Mancikova, Alberto Cascón, Mercedes Robledo y Cristina Rodríguez-Antona

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Resumen: La neuropatía periférica es la toxicidad limitante de dosis del paclitaxel, un fármaco quimioterapéutico ampliamente utilizado para el tratamiento de varios tumores sólidos. El efecto citotóxico del paclitaxel está mediado por su unión a la β-tubulina de los microtúbulos celulares. En este estudio investigamos la neuropatía inducida por paclitaxel en relación a variantes genéticas en genes de β-tubulina. Para ello, determinamos los niveles de expresión de ARNm de tres isotipos de β-tubulina (I, IVb y IIa) en linfocitos de 100 voluntarios sanos y secuenciamos la región promotora de estos genes, para identificar polimorfismos potencialmente reguladores de la expresión génica. Encontramos diferencias de expresión de hasta 63 veces en el contenido de ARNm del gen de la β-tubulina IIa (*TUBB2A*) y tres polimorfismos en su promotor, a -101, -112 y -157 del inicio de la traducción, que se correlacionaban con mayores niveles de ARNm. La tasa de transcripción del gen *TUBB2A,* medida mediante ensayos de luciferasa, aumentó con las variantes -101G>A y -112C>T, en total desequilibrio de ligamiento. Además, tras genotipar estos polimorfismos en 214 pacientes tratados con paclitaxel demostramos que estas variantes protegían de la neuropatía periférica inducida por paclitaxel (HR= 0.62, 95%CI= 0.42-0.93, P= 0.021, análisis multivariable). Finalmente se observó una correlación inversa entre la expresión del *TUBB2A,* medida mendiante *Affymetrix exon array,* y la apoptosis inducida por el paclitaxel en líneas celulares linfoblastoides (P= 0.001), lo cual apoya que una mayor expresión del gen *TUBB2A* confiere una menor sensibilidad al paclitaxel. En conclusión, este es el primer estudio que demuestra que polimorfismos reguladores de la expresión de la β-tubulina se asocian al riesgo de neuropatía inducida por paclitaxel.

Aportación personal: LJ Leandro-García participó en el diseño del estudio, llevó a cabo el aislamiento del ADNg y del ARNm, la retro-transcripción a ADNc y cuantificación mediante qRT-PCR. Asimismo, amplificó los promotores cercanos mediante PCR y llevó a cabo las transfecciones celulares y medida de actividad luciferasa, y participó en el genotipado de las variantes reguladoras y el análisis estadístico. Finalmente, LJ Leandro-García participó activamente en la discusión crítica de los resultados y fue el autor principal en la redacción del trabajo para su publicación.

Predictive Biomarkers and Personalized Medicine

Clinical Cancer Research

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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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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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 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.

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

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

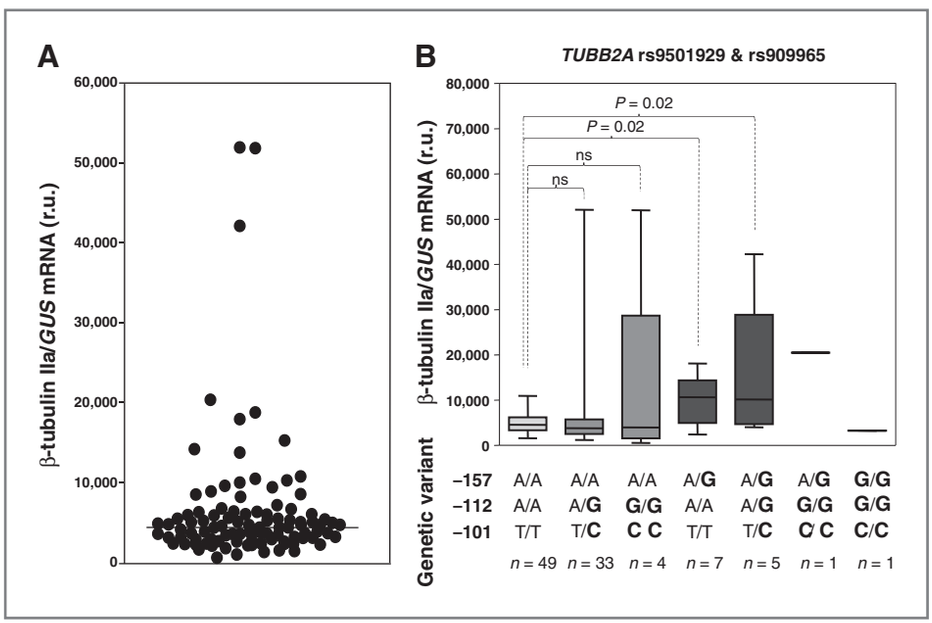

Figure 1. Interindividual variability in *TUBB2A* expression. A, *TUBB2A* mRNA content was measured by quantitative RT-PCR in 100 peripheral blood leukocytes from healthy donors, as described in Materials and Methods. The horizontal bar represents the median value. B, the healthy donors were genotyped 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 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.

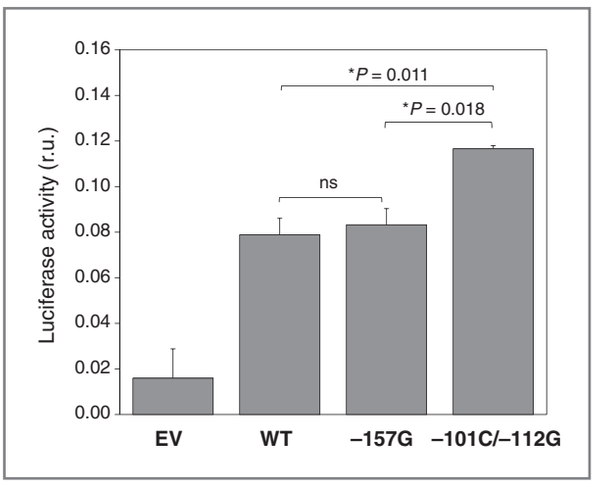

Figure 2. Effect of *TUBB2A* promoter variants on transcriptional activity. H1299 cells were transfected with pGL3-Basic (EV) and luciferase reporter plasmids with different polymorphisms in *TUBB2A* gene: pGL3B_WT (WT), pGL3B_−157G (−157G), and pGL3B_−101C/−112G (−101C/−112G). The cells were cotransfected 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.

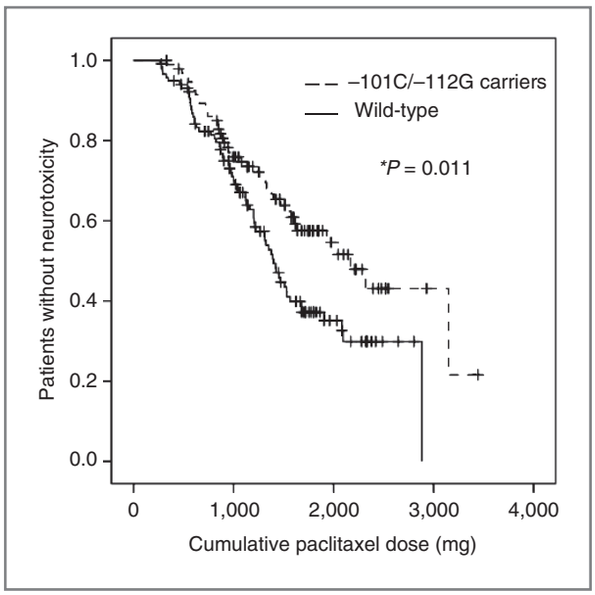

Figure 3. Kaplan–Meier analysis of cumulative dose of paclitaxel up to the development of grade 2 sensory peripheral neuropathy, according to −101C/−112G variants in *TUBB2A*. Patients treated with paclitaxel were grouped according to *TUBB2A* genotype. Those carrying 1 or 2 variant alleles had a significantly lower risk of paclitaxel-induced neurotoxicity. The *P* value shown corresponds to univariable log-rank test.

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

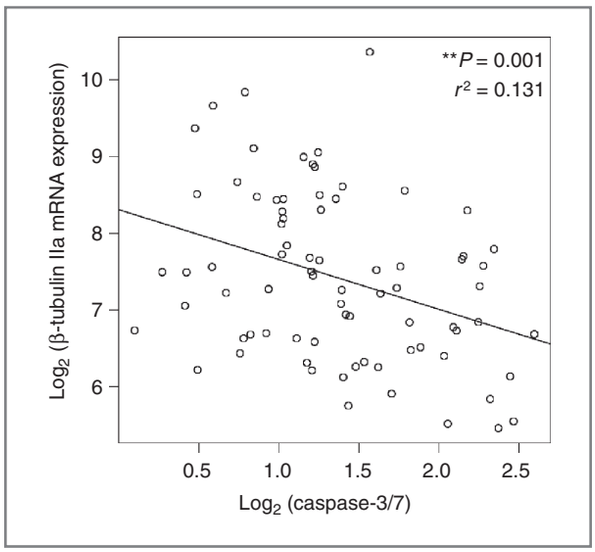

Figure 4. Inverse correlation between *TUBB2A* gene expression and paclitaxel-induced apoptosis. Lymphoblastic cell lines (CEU, $n = 77$) were treated with paclitaxel to measure caspase-3/7 activation (apoptosis), and Affymetrix exon array was used to measure *TUBB2A* expression in the same cell lines, 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L.J. Leandro-García, C. Jara, H. Gréen, E. Åvall-Lundqvist, H.E. Wheeler, M.E. Dolan, A. Cascón, M. Robledo

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L.J. Leandro-García, S. Leskelä, C. Jara, H. Gréen, H.E. Wheeler, M.E. Dolan, L. Inglada-Perez, M. Robledo, C. Rodríguez-Antona

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MATERIAL SUPLEMENTARIO

Supplementary Table 1. Oligonucleotides used for β-tubulins mRNA quantification and *TUBB2A* sequencing and cloning.

Technique	Forward oligonucleotide 5'-3'	Reverse oligonucleotide 5'-3'	Probe 5'-3'
qRT-PCR_β-tub I	ATACCTTGAGGCGAGCAAAA	CTGATCACCTCCCAGAACTTG	UPL #64
qRT-PCR_β-tub IIa	AAATATGTACCTCGGGCCATC	GTTATTCCCGGCTCCACTCT	UPL #50
qRT-PCR_β-tub IVb	TTGTCTACTTCCTCCTGCTTCC	CTGATCACCTCCCAAAACTTG	UPL #70
Sequencing_β-tub IIa	CAGCGGCAGCAGCAATAG	GATCTGGTTGCCGCACTG	
Cloning_β-tub IIa	CGGGCTCGAGATCGCGGCGTGAGCGGAGGA	TCTCAAGCTTGGTGCCGGCTGCGGAGCGGGT	

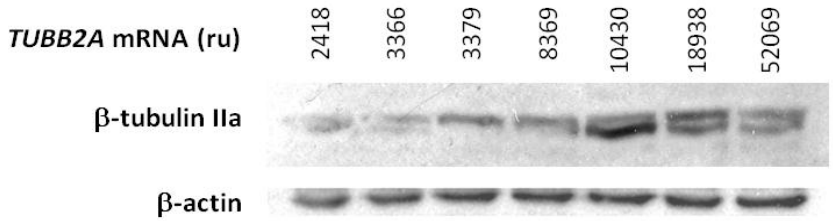
Supplementary Table 2. *TUBB2A* promoter variants in high and low β-tubulin IIa mRNA expression individuals.

Variants		β-tubulin IIa	
Location	**ID, nucleotide change**	**Low mRNA, n=9[a] (number variant alleles)**	**High mRNA, n=11[a] (number variant alleles)**
-58	not described, C>A	0	1
-91	rs13219681, G>A	1	5
-92	not described, G>A	1	0
-101[b]	**rs909964, T>C**	**5**	**10**
-112[b]	**rs909965, A>G**	**5**	**10**
-115	rs6915357, C>T	0	1
-157[b]	**rs9501929, A>G**	**1**	**7**

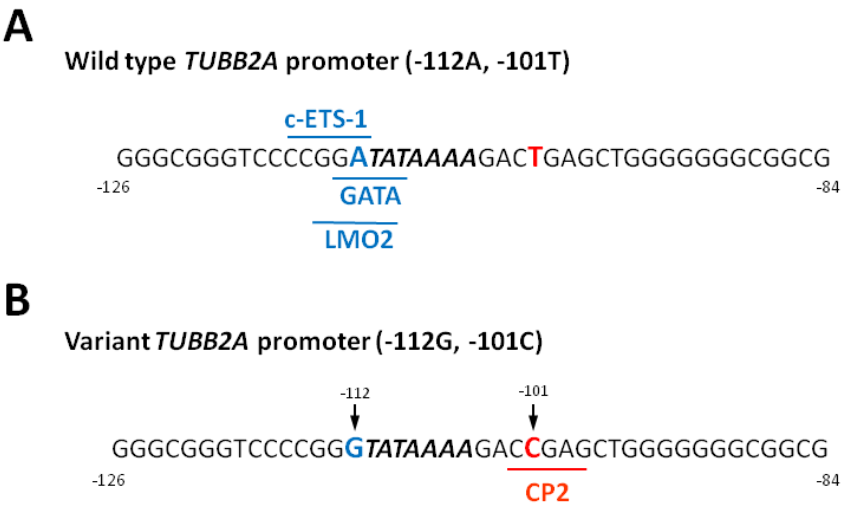
[a] The promoter region of *TUBB2A* was sequenced in 9 healthy volunteers with the highest and 11 with the lowest *TUBB2A* expression in lymphocytes (>10000 and <2500 *TUBB2A* mRNA relative units (r.u.), after normalization with the internal control GUS).

[b] These variants were selected for further evaluation.

Supplementary Figure 1. β-tubulin II protein expression in peripheral blood leukocytes. β-tubulin II protein was detected in peripheral blood leukocytes from healthy volunteers with low and high *TUBB2A* mRNA expression by western blot using an antibody specific for IIa and IIb isotypes (clone 7B9; Covance, Emeryville, CA, USA). β-actin was used as control in the western blot.



Supplementary Figure 2. Putative binding sites for transcription factors in *TUBB2A* proximal promoter. Schematic representation of putative transcription binding sites that could be altered by the -101 and -112 *TUBB2A* variants (MATCH™ public version 1.0, using Matrix Search for Transcription Factor Binding Sites; http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi, using as core match and matrix match cut-offs 0.875 and 0.700, respectively). **A.** Wild type *TUBB2A* promoter. **B.** -101 and -112 variant *TUBB2A* promoter. The TATA box is in bold; polymorphisms at -101 and -112 positions are marked with arrows. Transcription factors affected by -101 and -112 polymorphism are shown in red and blue, respectively. Nucleotide positions refer to the translation start site (ATG, +1).



ARTÍCULO 4: Hematologic β-tubulin VI isoform exhibits genetic variability that influences paclitaxel toxicity.

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*** Estos autores han contribuido igualmente.**

Aceptado para publicación en Cancer Res. 2012 Jul 17.

Resumen: Los microtúbulos celulares, compuestos de heterodímeros de α-β-tubulina, son esenciales para diversas funciones celulares, asimismo, son la diana terapéutica de diversas terapias oncológicas. Puesto que la β-tubulina VI es el isotipo hematológico prioritario, variaciones en esta proteína podrían alterar la acción mielosupresora de los agentes de unión a microtúbulos. En este estudio hemos determinado los patrones de expresión de la β-tubulina VI (codificada por el gen *TUBB1*) en distintas células sanguíneas e identificado polimorfismos codificantes potencialmente funcionales. La expresión de la β-tubulina VI fue alta en todas las células de la sangre examinadas y mostró una variabilidad interindividual considerable en linfocitos (de hasta 7 veces en el contenido de ARNm). En el gen *TUBB1* encontramos varios polimorfismos que codificaban para cambios de amino ácido: p.Q43P, p.T274M y p.R307H, y una variante rara de parada de la traducción p.Y55X. Expresamos estas variantes de forma estable en líneas celulares y determinamos si modificaban el efecto del paclitaxel en la estabilización de los microtúbulos. Observamos que la β-tubulina VI p.274M era significativamente más resistente al efecto del paclitaxel. Además, los pacientes portadores la variante p.T274M en la β-tubulina VI y tratados con paclitaxel tuvieron una trombocitopenia significativamente más baja que los pacientes homocigotos para la variante silvestre de este polimorfismo (P= 0.031). En conjunto, este estudio demuestra que la β-tubulina VI humana es un isotipo hematológico con una variación genética significativa que podría afectar la acción de mielosupresora de los agentes de unión a microtúbulos.

Aportación personal: LJ Leandro-García participó en el diseño del estudio, llevó a cabo el aislamiento del ADNg y del ARNm, amplificación, purificación y secuenciación de fragmentos de PCR, y participó en el genotipado de las variantes. Además, participó en el clonaje y la mutagénesis del *TUBB1*, en el establecimiento de líneas celulares que se expresaban de forma estable las variantes de la β-tubulina VI y en los ensayos de polimerización y análisis estadístico. Finalmente, LJ Leandro-García participó activamente en la discusión crítica de los resultados y fue uno de los dos autores principales en la redacción del trabajo para su publicación.

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Hematologic β-Tubulin VI Isoform Exhibits Genetic Variability That Influences Paclitaxel Toxicity

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Abstract

Cellular microtubules composed of α-β-tubulin heterodimers that are essential for cell shape, division, and intracellular transport are valid targets for anticancer therapy. However, not all the conserved but differentially expressed members of the β-tubulin gene superfamily have been investigated for their role in these settings. In this study, we examined roles for the hematologic isoform β-tubulin VI and functional genetic variants in the gene. β-tubulin VI was highly expressed in blood cells with a substantial interindividual variability (seven-fold variation in mRNA). We characterized DNA missense variations leading to Q43P, T274M, and R307H, and a rare nonsense variant, Y55X. Because variations in the hematologic target of microtubule-binding drugs might alter their myelosuppressive action, we tested their effect in cell lines stably expressing the different β-tubulin VI full-length variants, finding that the T274M change significantly decreased sensitivity to paclitaxel-induced tubulin polymerization. Furthermore, patients treated with paclitaxel and carrying β-tubulin VI T274M exhibited a significantly lower thrombocytopenia than wild-type homozygous patients ($P = 0.031$). Together, our findings define β-tubulin VI as a hematologic isotype with significant genetic variation in humans that may affect the myelosuppresive action of microtubule-binding drugs. A polymorphism found in a tubulin isoform expressed only in hemapoietic cells may contribute to the patient variation in myelosuppression that occurs after treatment with microtubule-binding drugs. *Cancer Res; 72(18); 1–9. ©2012 AACR.*

Introduction

Microtubules are ubiquitous and highly dynamic polymers of α-β-tubulin heterodimers indispensable for a variety of cellular functions such as structure maintenance, intracellular transport, cell signaling, migration, and mitosis. Several of the most common chemotherapeutic drugs, such as taxanes, vinca-alkaloids, and epothilones, base their mechanism of action on binding to microtubules and altering their dynamics, which leads to mitosis arrest and cell death (1, 2). The therapeutic target of these drugs is β-tubulin, which consists of 8 isotypes encoded by multiple genes that exhibit a tissue-specific expression. In a previous study, we showed that isotypes I, IVb, and V are constitutive, isotypes IIa, IIb, III, and IVa are neuronal, and isotype VI is hematopoietic cell-specific and was detected in platelets, lymphocytes, bone marrow, and spleen (3, 4). The β-tubulin isotypes are highly conserved and have similar sequences, however, the C-terminal region exhibits higher variability and seems to confer differences in microtubule polymerization and stability. The fact that rare mutations in β-tubulin IIb and III lead to a spectrum of severe neuronal disorders suggests specific functions that cannot be compensated by alternative isotypes (5, 6). β-Tubulin VI knockout mice studies proved a specialized role for this protein in platelet synthesis, structure, and function (7, 8).

Because of the crucial role of β-tubulin in cells, genetic variation is not allowed. However, the hematologic isotype VI is an exception, and a common missense polymorphism (Q43P) has been associated with an altered risk of cardiovascular disease by modulating platelet function and structure (9, 10). There is also a report on a rare β-tubulin VI missense variant (R318W) responsible for congenital macrothrombocytopenia (11). Other genetic variations in β-tubulin isotypes correspond to cell lines and lead to acquired resistance to microtubule-binding drugs (12–14). Variations in β-tubulin isotype composition in tumors have also been associated with decreased sensitivity to these drugs (15–18). Thus, polymorphisms in β-tubulin VI, leading to a variant protein or altered expression,

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might result in differences in the effect of β-tubulin–binding drugs among patients. Because of the hematologic role of β-tubulin VI, these polymorphisms could affect the action of the drugs in blood cells, leading to interpatient differences in myelosupression. However, at the moment, the expression of β-tubulin VI has not been well-established, and polymorphisms in this gene have not been investigated.

In this study, we characterized β-tubulin VI expression in hematologic cells and screened for genetic variation by sequencing the coding region of the β-tubulin VI gene (*TUBB1*). We generated cell lines stably expressing the variant β-tubulin VI proteins detected and conducted functional assays. Furthermore, the clinical implications of these polymorphisms were studied in patients with hematologic malignancies treated with paclitaxel.

Materials and Methods

Human samples and patients

cDNAs from 7 different blood fractions were obtained from BD Biosciences (Human Blood Fractions MTC Panel), peripheral blood lymphocytes, platelets, and neutrophils were isolated from healthy volunteers, and $CD34^-$ bone marrow cells were isolated from bone marrow aspiration following previously described procedures (19). Frozen samples from peripheral T-cell lymphomas were collected through the Spanish National Cancer Centre (Madrid, Spain) tumor bank network from pathology departments of different hospitals in Spain (20). The main characteristics of this human material and cell lines used for mRNA quantification are shown in Supplementary Table S1. For the estimation of allele and haplotype frequencies, we used DNA isolated from unrelated individuals from different ethnic groups, specifically, 481 Caucasians from Spain, 106 Asians from China, and 71 Africans from Tanzania.

Forty-nine patients with hematologic malignancies planned for autologous transplant (20 with lymphoma, 15 with multiple myeloma, and 14 with acute leukemia), who underwent mobilization of hematopoietic progenitor cells with paclitaxel 170 mg/m^2 i.v. by continuous infusion for 24 hours followed by 8 μg/kg s.c. recombinant human granulocyte colony-stimulating factor (rhG-CSF) daily, and that had leukapheresis product available, were included in the study (21). In this series, recruited between 1999 to 2008, the time elapsed from the last cytotoxic treatment was at least 3 weeks, to allow recovery of peripheral blood counts before paclitaxel administration. Peripheral blood counts were assessed on days 5, 7, and daily afterward. Maximal hematologic toxicity was retrospectively assessed and recorded according to the National Cancer Institute-Common Toxicity Criteria version 3. The characteristics of the patients, including age at treatment, previous treatments, state of disease at stem cell harvest, weeks from the last treatment, and baseline platelet counts at paclitaxel treatment initiation are provided in Supplementary Table S3.

RNA isolation and quantitative RT-PCR

RNA was extracted from blood cells, cell lines, and frozen tumoral tissue using TRI-reagent (Molecular Research Center Inc.) and the concentration quantified by using Nanodrop ND-1000 (Wilmington). One microgram of total RNA was reverse transcribed using Superscript II (Invitrogen) and an oligo dT14 primer following the manufacturer's instructions. The mRNA content of the different β-tubulin isotypes was quantified by quantitative reverse transcription-PCR (qRT-PCR) with the Sequence Detection System 7900HT (Applied Biosystems) using conditions, primers, and probes previously described (4). Normalization was carried out with the internal standard β-glucuronidase (*GUS*). Negative controls were present in all PCR series and assays were carried out in triplicates. The $\Delta\Delta C_t$ method (22) was used for the calculation of mRNA content.

DNA isolation, sequencing, and genotyping

Genomic DNA was isolated from the peripheral blood lymphocytes of the healthy volunteers and from the leukapheresis product of the hematologic patients using FlexiGene DNA Kit (QIAGEN), and the DNA concentration was determined using PicoGreen dsDNA quantification reagent (Invitrogen). For *TUBB1* sequencing, the exons and proximal promoter region were amplified by PCR using specific primers designed to avoid cross-reactions with other β-tubulin isotype genes/pseudogenes (Supplementary Table S2). 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 the *TUBB1* coding polymorphisms 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.

Platelet activation and aggregation assays

The effect of β-tubulin VI R307H polymorphism in platelet function was investigated by conducting aggregation assays in 6 healthy volunteers of known genotype (3 women homozygous for the wild-type allele and 2 women and 1 man homozygous for the variant allele). Platelet aggregation was measured in nonadjusted-citrated platelet-rich plasma obtained by centrifuging blood at 140 × *g* for 12 minutes. Platelets were stimulated with 6-mer thrombin receptor agonist at 0.78 to 25 μmol/L (Sigma-Aldrich), collagen at 0.125 to 2 μg/mL (Menarini Diagnostics), ADP at 0.16 to 0.5 μmol/L (Menarini Diagnostics), arachidonic acid at 0.4 to 1.6 mmol/L (Bio-Rad), and ristocetin at 0.75 to 1.25 mg/mL (Sigma-Aldrich). Changes in light transmission of platelet-rich plasma over baseline were recorded for 5 minutes using an Aggrecorder II aggregometer (Menarini Diagnostics).

Generation of cell lines expressing β-tubulin VI wild-type and variant proteins

We amplified the full coding sequence of *TUBB1* using specific primers that introduced *Nhe*I and *Not*I cleavage sites (Supplementary Table S2) and cDNA from peripheral blood lymphocytes that carried in homozygosity the wild-type *TUBB1* coding region. The PCR product was cloned into pIRESpuro2 vector (Clontech) to generate pIRESpuro2_TUBB1 wild-type

plasmid. By means of the GeneTailor Site-Directed Mutagenesis System (Invitrogen) and following the manufacturer's indications and adequate primer pairs (Supplementary Table S2), the pIRESpuro2_TUBB1 wild-type construct was used to generate plasmids that contained *TUBB1* sequence with variants encoding for the following protein changes: Q43P, Y55X, T274M, R307H, and T274M/R307H. Correct *TUBB1* sequence and lack of artifacts was confirmed by sequencing.

MCF7 breast cancer cells were provided by Dr M. Esteller (IDIBELL, Barcelona, Spain). MCF7 cells were chosen for heterologous expression of β-tubulin VI based on their lack of endogenous expression of this protein and sensitivity to microtubule-binding drugs. MCF7 cells were transiently transfected with the different *TUBB1* constructs and the empty vector by using Lipofectamine-2000 (Invitrogen) following the manufacturer's instructions. To generate stable cell lines, the different *TUBB1* constructs and the empty vector were electroporated using the Gene Pulser XCell Electroporation System (Bio-Rad) into cells and 24 hours later, 0.5 μg/mL of puromycin (Sigma-Aldrich) was added for selection. Puromycin-resistant clones were analyzed for β-tubulin VI expression by Western blot analysis. Control cells were also selected with puromycin, all resistant empty vector clones pooled together. Stable transfectants were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS, penicillin, streptomycin, and puromycin at a concentration of 0.4 μg/mL.

β-Tubulin VI protein detection

For Western blotting, total cell extracts from cell lines were separated by using the Criterion XT Gels Bis-Tris 10% and the Criterion electrophoresis cell (Bio-Rad) and transferred to polyvinylidene fluoride membranes (Immobilon-P Membrane, Millipore) using the Trans Blot Semi-Dry Blotter (Bio-Rad). Protein concentration was measured by using Bio-Rad Protein Assay (Bio-Rad), and equal loading of proteins was verified by Ponceau S staining. The membranes were blocked and then incubated with a rabbit polyclonal antibody specific for human β-tubulin VI (from Dr. Paraskevi Giannakakou; ref. 3), a mouse antibody recognizing all β-tubulin isotypes (clone 2.1, Sigma; 1:1,000 dilution), or a rat anti-α–tubulin (clone YL1/2; Chemicon International; 1:1,000 dilution). After washing, the membranes were incubated with the corresponding secondary antibody: goat anti-rabbit (Alexa Fluor 680 nm, Molecular Probes; 1:15,000 dilution), goat anti-mouse (DyLight 800, Thermo Scientific; 1:10,000 dilution), or donkey anti-rat (IRdye 800 nm, Rockland Immunochemicals; 1:15,000 dilution). The fluorescence signal was detected using the Odyssey infrared imaging system (LI-COR).

Immunofluorescence microscopy was conducted as previously described (23). In brief, cells were cultivated on coverslips overnight and fixed the next day with 4% paraformaldehyde, permeabilized with Triton-X-100 0.5% in TBS, and blocked with 10% goat serum. The same primary antibodies as those described for Western blotting were used. Specific secondary antibodies, Alexa-rabbit-405 nm and Alexa-rat 568 nm, were used. Cells were imaged using a Zeiss LSM 5 LIVE confocal microscope using a ×40/1.3 EC Plan Neofluar objective, a ×63/1.4 Plan APOCHROMAT objective, and ×100/1.4 Plan APOCHROMAT objectives. All images were acquired and analyzed using Zeiss LSM 5 LIVE software.

Tubulin polymerization assay

Quantitative drug-induced tubulin polymerization assays were conducted as previously described (12). In brief, cells were grown in 24-well plates overnight and incubated for 6 hours with paclitaxel at concentrations of 0, 10, 100, and 1,000 nmol/L. The cells were washed twice with PBS before lysis at 37°C for 5 minutes in the dark with 100 μL of hypotonic buffer (20 mmol/L Tris-HCl pH 6.8, 1 mmol/L $MgCl_2$, 2 mmol/L EGTA, and 0.5% Nonidet P40) containing protease inhibitors (Protease Cocktail inhibitor tablets; Roche). The lysates were transferred to 1.5 mL Eppendorf tubes, rinsed with 100 μL of hypotonic buffer, mixed by vortexing, and centrifuged at 14,000 rpm for 10 minutes at room temperature. The 200 μL supernatants containing soluble (cytosolic) tubulin were transferred to another tube, and pellets containing polymerized (cytoskeletal) tubulin were resuspended in 200 μL of hypotonic buffer. The cytosolic and cytoskeletal fractions were each mixed with electrophoresis sample buffer, sonicated, boiled, and analyzed by immunoblotting. The percentage of polymerized tubulin was determined by dividing the densitometry value of polymerized tubulin by the total tubulin content (the sum of the densitometry values of soluble and polymerized tubulin) in at least 3 independent experiments.

Statistical analysis

The myelotoxicity of the patients was analyzed taking into account gender, age at paclitaxel treatment, type of disease, previous treatments, state of disease at stem cell harvest, weeks from last treatment, and baseline platelet counts at the initiation of paclitaxel and genotypes. For the analysis, quantitative variables were dichotomized according to the median. To include previous chemotherapy as a covariable, we used a bone marrow toxicity scoring system, which takes into account drugs and number of chemotherapy cycles used (24). This chemotherapy score correlates with damage to the stem cell pool caused by previous chemotherapy and with the ability to mobilize hematopoietic cells (25, 26). Acute leukemias were excluded from this analysis because, although treatments only include low/medium myelotoxic drugs and they have low chemotherapy score, the intense dosages in these protocols cause prolonged aplasias (Supplementary Table S3). The χ^2 test and Fisher exact test were used to compare the hematologic toxicity grade of patients with clinical variables and genotype. To correct for the possible effect of previous treatments on the thrombocytopenia, the Mantel–Haenszel test was used. Nominal 2-sided P values < 0.05 were considered statistically significant. All statistical analyses were carried out using SPSS software package version 17.0 (SPSS).

Results

β-Tubulin isotype VI has a broad and variable expression in blood cells

We previously showed that β-tubulin VI expression was exclusively found in hematopoietic lineage cells, specifically, in

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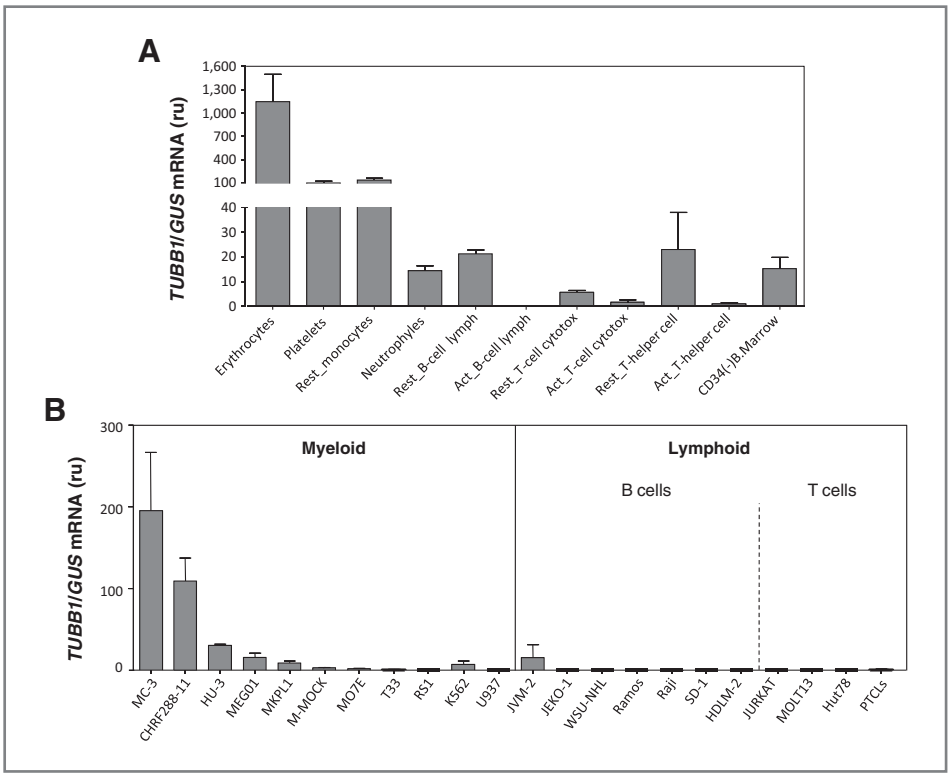

Figure 1. β-tubulin VI mRNA content in hematologic cells. qRT-PCR was used to determine β-tubulin VI (*TUBB1*) mRNA quantities in different cells. A and B, *TUBB1* mRNA content in different nontumoral human hematologic cells (A) and in different cell lines and tumors of myeloid and lymphoid origin (B). mRNA content is compared with platelets expression (100%). ru, relative units; Rest, resting; Act, activated.

platelets, lymphocytes, bone marrow, and spleen; however, a detailed characterization of β-tubulin VI expression has not been conducted. Here, we show that β-tubulin VI mRNA was detected in all blood samples tested with the highest expression corresponding to erythrocytes, platelets, and monocytes (cells of myeloid origin; Fig. 1A). To further investigate the expression of β-tubulin VI, we also determined β-tubulin VI mRNA content in hematologic malignancies by studying tumoral cell lines and samples. Again, cells of myeloid origin showed higher expression than those of lymphoid origin, with the megakaryocytic MC-3 and CHRF288-11 cell lines showing the highest expression levels (Fig. 1B).

To confirm the relevance of β-tubulin VI in blood cells, we studied its expression in peripheral blood lymphocytes from 100 healthy volunteers. Figure 2A shows that isotype VI was the major β-tubulin component of lymphocyte microtubules, followed by β-tubulins IVb and I, accounting for 82, 10, and 7.5% of the total β-tubulin content, respectively. Interestingly, β-tubulin VI mRNA content was subjected to a substantial interindividual variability (7.2-fold variation in expression; Fig. 2B). To substantiate this variability, we also measured the expression of the ubiquitous and highly conserved β-tubulins IVb and I in the same samples, finding a 2.5- and 2.2-fold variation in mRNA content, respectively (data not shown).

TUBB1 gene has common missense polymorphisms

To determine whether *TUBB1* gene could differentiate from the other β-tubulin isotypes and be subjected to common genetic variability, we sequenced the 4 exons and part of the 5′ and 3′ untranslated region of *TUBB1* in genomic DNA from 20 unrelated Caucasians. In addition to the already studied missense polymorphisms, rs463312 (Q43P) and rs415064 (Q43H), we found 2 other nucleotide changes leading to amino acid changes, rs35565630 (T274M) and rs6070697 (R307H), and one resulting in a premature stop codon (c.585G>A; Y55X). To determine the allelic frequency of these genetic variations, we conducted genotyping in a series of unrelated Caucasians, Asians, and Africans (Table 1). The nucleotide change leading to an early stop codon was only detected in the initial case used for sequencing. Regarding the other variants, the polymorphism leading to R307H was the most frequent, followed by those corresponding to Q43P, Q43H, and T274M, this latter not detected in Asians. When we established the haplotypes formed by these missense variants (Table 1), we found that, as previously observed, rs463312 and rs415064 were in total linkage disequilibrium resulting exclusively in Q43P. rs35565630 was found only in the presence of rs6070697, thus, encoding a protein with 2 amino acid changes (T274M and R307H), whereas a relatively high number of cases carried only rs6070697 (R307H).

Concerning the putative effect of the *TUBB1* coding variants, previous results have shown that Q43P affects cardiovascular risk through an alteration of platelet aggregation. To determine whether the newly described missense polymorphisms could also alter platelets function, we isolated DNA from 100 healthy volunteers and identified individuals carrying the variants in homozygosity. For T274M, we could not find homozygous variant individuals, thus, we conducted platelet activation and aggregation assays only for rs6070697 (R307H) and wild type homozygous individuals; however, we did not detect significant differences (data not shown). In addition, we tested

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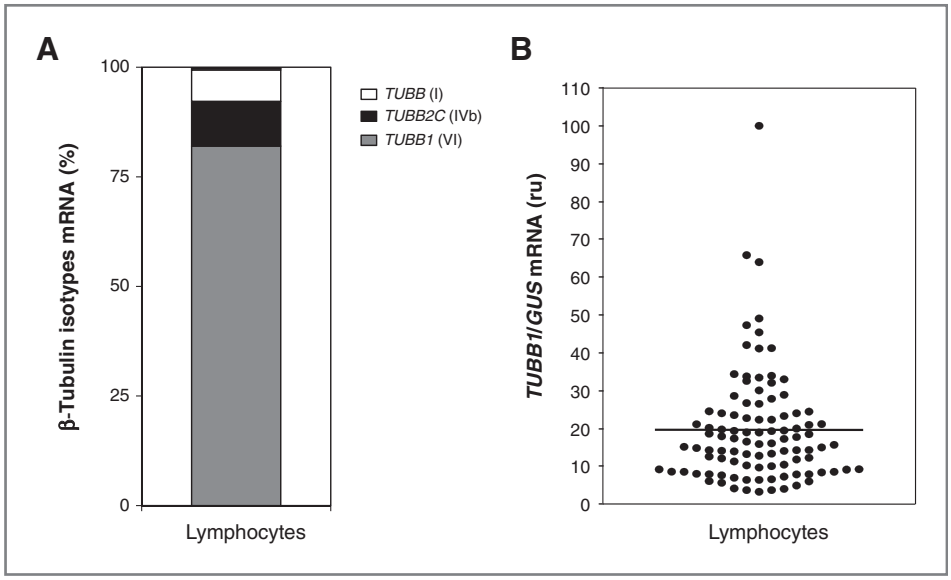

Figure 2. β-tubulin isotypes relative expression in lymphocytes and interindividual variability in β-tubulin VI mRNA expression. A, β-tubulin isotypes mean relative mRNA expression in peripheral blood lymphocytes from 100 healthy volunteers (*TUBB1*: 82.00%; *TUBB2C*: 10.00%; *TUBB:* 7.10%; *TUBB6:* 0.35% and *TUBB2A:* 0.27%. mRNA from *TUBB3, TUBB2B*, and *TUBB4* below detection limits). B, *TUBB1* mRNA content was quantified by qRT-PCR in peripheral blood lymphocytes isolated from 100 healthy volunteers. ru, relative units.

whether the missense polymorphisms could be associated with an altered *TUBB1* mRNA content and thus explain the inter-individual variation found in the expression of this gene (Fig. 2B); however, none of the polymorphisms was significantly associated with an altered mRNA content (data not shown). Sequencing of *TUBB1* proximal promoter region in individuals with maximal and minimal mRNA content did not identify polymorphisms associated with the expression of the gene.

β-Tubulin VI T274M alters paclitaxel effect on tubulin polymerization and is associated with decreased thrombocytopenia in patients treated with paclitaxel

To test the effect of the *TUBB1* missense polymorphisms, we carried out transient transfections using appropriated expression vectors in MCF7 cells. These cells are derived from a solid tumor and do not express β-tubulin VI protein. As shown in Fig. 3A and B, the exogenously expressed β-tubulin VI proteins were correctly produced and colocalized with α-tubulin, indicating a correct incorporation into the cytoskeleton of the transfected cells. Cells stably expressing the different β-tubulin VI proteins were used to study tubulin polymerization after treatment with increasing amounts of paclitaxel. We found that in control cells, transfected with the empty vector, most of the cellular β-tubulin shifted to a polymerized form at 100 nmol/L paclitaxel. This same effect was also appreciated in cells expressing wild-type, Q43P, and R307H β-tubulin VI proteins. However, cells expressing T274M and T274M/R307H variant proteins had significantly higher amounts of soluble

Table 1. Genetic variants in *TUBB1* coding region and common haplotypes

Variants in coding region	Amino acid change	Caucasians ($N = 481$)		Asians ($n = 106$)		Africans ($n = 71$)	
		Frequency[a]	n[b]	Frequency	n	Frequency	n
rs463312	Q43P	0.042	130	0.086	105	0.029	68
rs415064	Q43H	0.042	130	0.086	105	0.029	68
—	Y55X	0.001	451	0.000	106	0.000	71
rs35565630	T274M	0.036	481	0.000	105	0.014	71
rs6070697	R307H	0.192	453	0.107	98	0.123	69
Haplotypes[c]	**Amino acid changes**	**Frequency**		**Frequency**		**Frequency**	
Hap_AGCCG	None	0.791		0.823		0.846	
Hap_AGCCA	R307H	0.146		0.107		0.110	
Hap_CCCCG	Q43P	0.037		0.069		0.029	
Hap_AGCTA	T274M/R307H	0.023		0.000		0.013	

[a]Frequency for the different alleles and haplotypes.
[b]Number of individuals used in each determination.
[c]Haplotypes estimated with frequencies >1%. The nucleotides correspond to rs463312, rs415064, the nucleotide change leading to Y55X, rs35565630, and rs6070697.

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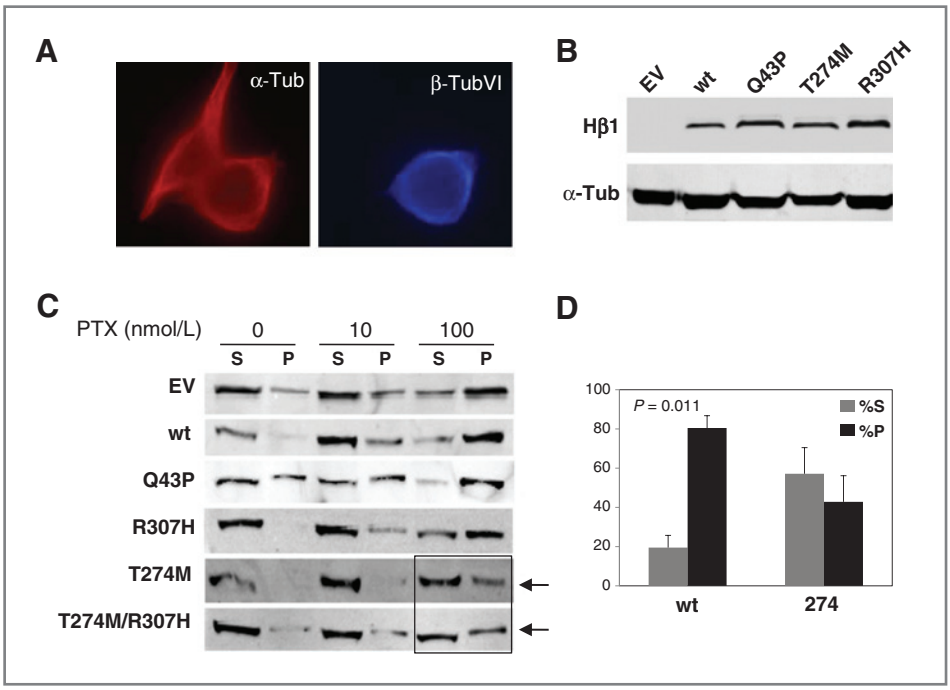

Figure 3. Cells expressing β-tubulin VI T274M variant protein are less sensitive to the effect of paclitaxel on tubulin polymerization. A, confocal microscopy images of β-tubulin VI transient transfection in MCF7 cells. α-Tubulin and β-tubulin VI staining are visualized in red (568 nm) and blue (405 nm), respectively. B, β-tubulin VI protein detection by Western blot analysis in MCF7 cells stably expressing the different variants and those transfected with the empty vector (EV). C, MCF7 cells stably expressing the different β-tubulin VI variants were treated with paclitaxel (PTX) for 6 hours. Control- and paclitaxel-treated samples were lysed and the polymerized (P) and the soluble (S) tubulin fractions were separated by centrifugation, loaded on adjacent lanes in SDS-PAGE, and immunoblotted with an antibody against total β-tubulin. No statistically significant differences were found for any of the variant proteins, except for those with T274M. Arrows indicate the lanes with significant differences. D, the percentage of polymerized (%P) and soluble (%S) tubulin was determined by dividing the densitometric value of polymerized (P) and soluble (S) tubulin by the total tubulin content (P + S). The bars in the graph represent the mean values of 3 different experiments.

β-tubulin at 100 nmol/L paclitaxel ($P = 0.011$; see Fig. 3C and D). These results suggest that β-tubulin VI T274M variant protein is less sensitive to the polymerization effect of paclitaxel.

Because β-tubulin VI is a hematologic isotype, we investigated whether β-tubulin VI T274M patients could be less sensitive to the myelosuppressive effect of paclitaxel. To determine this, we genotyped a series of 49 patients with nonsolid tumors scheduled for autologous transplant treated for mobilization of hematopoietic progenitor cells with paclitaxel 170 mg/m^2 i.v. by continuous infusion for 24 hours, followed by 8 μg/kg s.c. rhG-CSF daily. With this highly myelosupressive protocol, grades III and IV leukocytopenia and thrombocytopenia occurred in 55% and 43% of patients, respectively. We did not find a statistically significant association between clinical variables (e.g. type of disease, previous treatments, state of disease at stem cell harvest, weeks from last treatment, and platelet counts at baseline before the initiation of treatment with paclitaxel) and the hematologic toxicity of the patients; however, we found a statistically significant effect for T274M polymorphism. Heterozygous T274M patients showed significantly lower thrombocytopenia than homozygous wild-type subjects. The 2 T274M patients exhibited grade I toxicity, whereas 85% of homozygous wild-type patients developed higher thrombocytopenia grades (II, III, or IV; $P = 0.031$; Fig. 4). The association was also significant (Mantel–Haenszel test, $P = 0.028$) when the previous chemotherapy treatments were taken into account. No significant association was found between T274M and neutropenia. We also genotyped the patients for Q43P and R307H variants and, as expected, no differences in thrombocytopenia and neutropenia were found (data not shown).

Discussion

In this study, we show that β-tubulin VI is a hematology-specific isotype subjected to a substantial genetic difference that is likely contributing to the interindividual variability observed in the myelosuppressive effect of paclitaxel. A thorough characterization of β-tubulin VI expression in cells of hematopoietic lineage revealed a high β-tubulin VI expression not only in lymphocytes and platelets, as previously described (3, 4), but also in most blood cells and several hematologic malignancies (Fig. 1). These data and the lack of β-tubulin VI in nonhematopoietic tissues (4) indicate that this is a hematology-specific isotype, and hence a major target mediating the hematologic toxicity of the β-tubulin–binding drugs. In contrast, the low expression of this isotype in hematologic malignancies of lymphoid origin does not support a major role of the β-tubulin VI in the physiopathology of these diseases. Furthermore, we found substantial interindividual variability in

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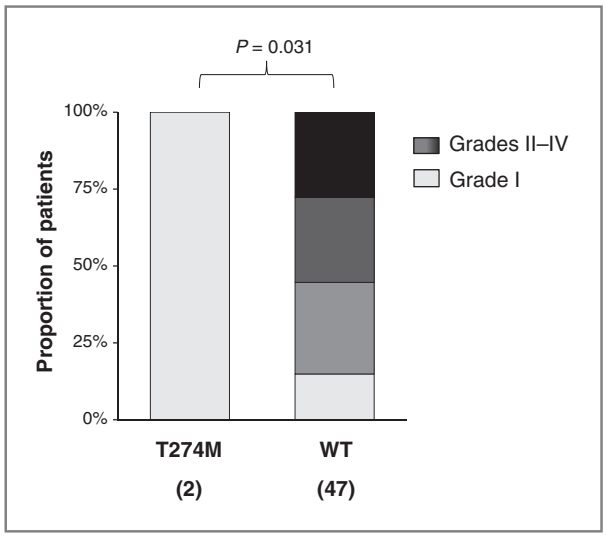

Figure 4. Patients that are carriers of β-tubulin VI T274M polymorphism develop less thrombocytopenia after paclitaxel treatment. Patients treated with paclitaxel 170 mg/m^2 i.v. by continuous infusion for 24 hours were genotyped and the thrombocytopenia grade developed after paclitaxel treatment (grade I vs. grades II, III, or IV) was compared with the homozygous wild-type patients using Fisher exact test.

β-tubulin VI mRNA content and common amino acid changes affecting *TUBB1* gene (Fig. 2 and Table 1).

So far, β-tubulin isotype genes have been considered highly conserved (27); however, here we prove that β-tubulin VI differentiates in this respect from the rest of isotypes and exhibits common variation in the coding region. β-tubulin VI Q43P was previously described and associated with a reduced risk of arterial thrombosis and an increased risk of intracerebral hemorrhage by modulating the platelet function and structure (9, 10). In addition, a rare β-tubulin VI variant, R318W, was described in a case of congenital macrothrombocytopenia (11). In this study, a nonsense variant (Y55X) was detected in heterozygosity; however, no deviations from normal ranges were found in the hemograms of this individual. So far, all the β-tubulin pathogenic variants are missense mutations (5, 6, 11), suggesting that early truncated proteins, that do not integrate into microtubules, might not be as damaging as variant proteins that lead to altered dynamic instability of microtubules (5, 6). This is in agreement with the lack of Y55X phenotype. The R307H polymorphism is located in a conserved region encoding β-tubulin VI M-loop; however, computational algorithms (PolyPhen and SIFT) predicted a benign variant and we found no significant effects on platelet activation and aggregation. T274M change also affects a conserved amino acid in the M-loop and was predicted as probably damaging by PolyPhen and SIFT; however, we detect this variant in the general population, suggesting it will not substantially alter its function. In regard to the microtubule-targeted drugs, residue 274 is located near the taxanes binding site (28).

We found that cells stably expressing β-tubulin VI 274M variant, alone or in combination with 307H, were less sensitive to the polymerizing effect of paclitaxel compared with control cells transfected with the empty vector and those expressing β-tubulin VI wild-type, 43P, and 307H variant proteins (Fig. 3). Human β-tubulin isotypes are essentially invariant within paclitaxel-binding site and, specifically, residue 274 is evolutionarily conserved in all vertebrate β-tubulins and all known β-tubulin isotypes in these organisms (29). Interestingly, residue 274 clusters in a space that has been identified as essential for interaction of paclitaxel with tubulin and an acquired mutation in residue 274 of β-tubulin I (T274I) renders cell lines resistant to paclitaxel and epothilone A (28, 30). These data suggest that variants affecting threonine 274 can lead to impaired drug binding and, thus, patients carrying the T274M variant could be resistant to the myelosuppressive effects of these microtubule-binding drugs. To investigate the impact of this polymorphism in patients and its potential as a marker of hematologic toxicity, we worked with a series of patients treated with paclitaxel. Because paclitaxel hematologic toxicity greatly depends on the time used for drug infusion (31), we decided to work with an outstanding set of 49 patients treated with a highly myelosuppressive protocol used for mobilization of hematopoietic progenitor cells. Although only 2 patients were T274M carriers, they showed a statistically significant lower grade of thrombocytopenia ($P = 0.031$; Fig. 4), suggesting that *TUBB1* T274M conferred protection against this paclitaxel toxicity. It is important to highlight that the T274M carriers had similar characteristics than the noncarriers in terms of age, disease state, base line platelets, and chemotherapy score (Supplementary Table S3). With respect to the type of disease, both were patients with myeloma with treatments that have an important effect on platelet production. Concerning the interval from last chemotherapy, the two carriers were among the patients with shorter times. No significant associations were found for leukocytopenia, but the results are inconclusive, because patients were treated with granulocyte colony-stimulating factor (G-CSF) to stimulate the production of neutrophils, and this is an important confounding factor. The effect found for T274M in thrombocytopenia risk is supported by the critical role that β-tubulin VI plays in platelets, showed both in knockout mice (7) and in patients carrying a mutation in this gene (11). Although in these previous studies, no pathogenic phenotypes were reported in neutrophils, we detected high contents of β-tubulin VI in these cells (Fig. 1A). This, together with the fact that the microtubule-binding agent 2-methoxyestradiol does not have an effect on β-tubulin VI (3) and does not result in any type of myelosupression (32), supports a function of this isotype also in neutrophils and consequently in drug induced neutropenia. The similar structure of paclitaxel and docetaxel, which share the binding domain with epothilones, and the fact that cell lines expressing β-tubulin I T274I are resistant to all of these agents (28), warrants further studies to investigate the role of T274M in the hematologic toxicity of these drugs. It can be estimated that approximately 7% of Caucasians carry T274M and might show differences in myelosupression.

In summary, this is the first study showing that β-tubulin VI is a hematology-specific isotype that differentiates from other β-tubulin genes by relevant genetic and expression variability. In addition, we show that β-tubulin VI T274M decreases the effect of paclitaxel on tubulin polymerization and, although

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validation in independent patient series is required, we provide data supporting that it might constitute a marker of hematologic toxicity induced by paclitaxel.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: L.J. Leandro-Garcia, S. Leskela, C. Rodriguez-Antona
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L.J. Leandro-Garcia, S. Leskela, L. Inglada-Perez, J. Rivera, M.L. Lozano, C. Rodriguez-Antona
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MATERIAL SUPLEMENTARIO

Supplementary Table 1. Characteristics of the human cells used for qRT-PCR.

Sample	Source/ Cell line type	Characteristics
Erythrocytes	1 Caucasian, age 27	Non-tumoral cells
Platelets	Pool from 12 Caucasians, ages 25-45	Non-tumoral cells
Neutrophyles	1 Caucasian, age 27	Non-tumoral cells
CD34(-) Bone Marrow	Unknown	Non-tumoral cells
Resting CD14+ (monocytes)	Pool from 19 Caucasians, ages 22-54	Non-tumoral cells
Resting CD19+ (B-cell lymphocytes)	Pool from 31 Caucasians, ages 19-56	Non-tumoral cells
Activated CD19+ (B-cell lymphocytes)	Pool from 7 Caucasians, ages 21-49	Non-tumoral cells
Resting CD8+ (T-cell cytotoxic lymph)	Pool from 33 Caucasians, ages 24-51	Non-tumoral cells
Activated CD8+ (T-cell cytotoxic lymph)	Pool from 8 Caucasians, ages 22-48	Non-tumoral cells
Resting CD4+ (T-cell helper lymph)	Pool from 6 Caucasians, ages 18-52	Non-tumoral cells
Activated CD4+ (T-cell helper lymph)	Pool from 8 Caucasians, ages 20-42	Non-tumoral cells
MC-3	Chronic myeloid leukemia in blastic crisis, M7	Tumoral cell line
CHRF288-11	Pediatric acute myeloid leukemia, M7	Tumoral cell line
HU-3	Acute myeloid leukemia, M7	Tumoral cell line
MEG01	Chronic myelocytic leukemia in blastic crisis, M7	Tumoral cell line
MKPL1	Myeloid cell line, M7	Tumoral cell line
M-MOCK	Pediatric acute myeloid leukemia, M7	Tumoral cell line
MO7E	Pediatric acute myeloid leukemia, M7	Tumoral cell line
T33	Chronic myelocytic leukemia in blastic crisis, M7	Tumoral cell line
RS1	Pediatric acute myeloid leukemia, M7	Tumoral cell line
K562	Chronic myelocytic leukemia in blastic crisis, M6	Tumoral cell line
U937	Histiocytic lymphoma, B-cell	Tumoral cell line
JVM-2	Mantle cell lymphoma, B-cell	Tumoral cell line
JEKO-1	Mantle cell lymphoma, B-cell	Tumoral cell line
WSU-NHL	B-cell lymphoma	Tumoral cell line
Ramos	Burkitt's lymphoma, B-cell	Tumoral cell line
Raji	Burkitt's lymphoma, B-cell	Tumoral cell line
SD-1	Acute lymphoblastic leukemia, B-cell	Tumoral cell line
HDLM-2	Hodking lymphoma, B-cell	Tumoral cell line
JURKAT	Actue T-cell leukemia	Tumoral cell line
MOLT13	T-cell lymphoma	Tumoral cell line
Hut78	T-cell lymphoma	Tumoral cell line
PTCL	Pool from 6 Peripheral T-cell lymphomas	Tumoral cells

Supplementary Table 2. Oligonucleotides used for *TUBB1* sequencing, cloning and mutagenesis.

Technique	Amplicon	Forward oligonucleotide 5'-3'	Reverse oligonucleotide 5'-3'
Sequencing	Promoter	TTGGAGAATTCAGGGTCTGG	GTGTGAGCCCAACACAACTG
	Exon 1	GGACACACCCTTGGTCACAT	TAAAGCCCAAAGGCATTGTC
	Exon 2	AACAGGCTTGGGAATGCTAA	GGGACCCACCACTCTAGGAT
	Exon 3 & 4a	GGGATTGTTGTTGGGGTAAA	ACCGGAGGGAGGTGGTTAT
	Exon 4b	TTCCGTACCCTGAAGCTGAC	GCAGAGTGGGTTTTGGAGTG
Cloning	Coding region	CAGA*GCTAGC*ATGCGTGAAATTGTCCATATTCAGATT	CTTC*GCGGCCGC*TTAATGTCCCTTATCTTCTGGCTC
Mutagenesis	Q43P	GGGGCCTCGGCCTTGCCCCTGGAGAGAATC	GCAAGGCCGAGGCCCCGCGGTCGCTCC
	Y55X	GTGTACTACAACGAAGCCTAAGGTAGGAAAT	TAGGCTTCGTTGTAGTACACGCTGATTCTCT
	T274M	GCTTTGCCCCACTCATGGCCCAGGGC	TGAGTGGGGCAAAGCCGGGCATAAAGAAGTGC
	R307H	CCTGTGACCTCCGCCATGGCCGCTAC	GGCGGAGGTCACAGGCAGCCATGGTATTG

Supplementary Table 3. Clinical characteristics of the patients.

Patient ID	Gender[a]	Age (y)	Disease[b]	Stage	Disease state[c]	Baseline platelets[d] (10^9/L)	Previous treatments[e]	Chemotherapy score[f]	Weeks from last treatment	rs35565630
10-452	M	38	ALL	na	CR	148	Pethema LAL-03AR	Na (11)	5	C/C
10-537	M	44	ALL	na	CR	248	Pethema LAL-93AR + (hyperCVAD x4)	Na (26)	5	C/C
10-518	M	15	ALL	na	CR	100	Pethema LAL-RI96	Na (10)	3	C/C
10-468	F	46	AML	na	CR	60	IDICE + cytarabine-mitoxantrone	Na (6)	24	C/C
10-454	M	51	AML	na	CR	61	IDICE + cytarabine-mitoxantrone	Na (6)	47	C/C
10-470	F	41	AML	na	CR	82	Idarubicine-cytarabine + mitoxantrone-cytarabine	Na (4)	16	C/C
10-529	F	51	AML	na	CR	96	IDICE x2 + cytarabine-mitoxantrone	Na (10)	4	C/C
10-451	M	68	AML	na	CR	104	IDICE + cytarabine-mitoxantrone	Na (6)	12	C/C
10-467	F	44	AML	na	CR	115	IDICE x2 + cytarabine-mitoxantrone	Na (10)	12	C/C
10-461	M	61	AML	na	CR	121	IDICE + cytarabin-mitoxantrone	Na (6)	8	C/C
10-473	M	33	AML	na	CR	133	IDICE + cytarabine-mitoxantrone; FLAG x2	Na (6)	11	C/C
10-455	M	55	AML	na	CR	151	IDICE + cytarabine-mitoxantrone	Na (6)	10	C/C
10-449	M	22	AML	na	CR	155	IDICE + cytarabine-mitoxantrone	Na (6)	15	C/C
10-463	M	15	AML	na	CR	166	Daunorubicin, cytarabine; doxorubicin, cytarabine, prednisone, thioguanine; mitoxantrone, cytarabine; FLAG-IDA x2	Na (10)	8	C/C
10-543	M	22	AML	na	CR	180	IDICE + cytarabine-mitoxantrone	Na (6)	6	C/C
10-546	M	46	HL	IVB	CR	174	CMOPP x13; ABVD x4	146	3	C/C
10-453	F	26	HL	IIA	PD	220	ABVD x6; DHAP x1; MiniBEAM x2	46	10	C/C
10-474	M	60	MM	IIIA	PR	80	Thalidomide-Dexamethasone x4; Bortezomib-Cyclophosphamide-Dexamethasone x3	6	3	C/C
10-547	M	59	MM	IIIA	PR	105	Melphalan-Prednisone x5; RT 30 Gy	20	6	C/C
10-548	M	56	MM	IIIA	PR	107	VCMP/VBAD x6; IFN	42	60	C/C
10-545	F	51	MM	IIIA	PR	149	VBMCP/VBAD x8	72	4	C/C

Supplementary Table 3. Clinical characteristics of the patients (continued).

10-525	F	66	MM	IIA	PR	151	VCMP/VBAD x6	42	5	C/C
10-511	**M**	**61**	**MM**	**IIIA**	**CR**	**165**	**Melphalan-Prednisone x5; VAD x3**	**29**	**4**	**C/T**
10-466	M	63	MM	IIA	CR	168	VBCMP/VBAD x4; bortezomib x2	28	10	C/C
10-531	**F**	**58**	**MM**	**IIB**	**PR**	**202**	**VCMP/VBAD x6**	**42**	**4**	**C/T**
10-475	M	52	MM	IIIA	PR	224	VAD x4	12	10	C/C
10-482	F	62	MM	IIA	CR	225	RT 400 cGy+ VBCMP/VBAD x4 + bortezomib x2	36	9	C/C
10-472	M	62	MM	IIIA	PR	251	VCMP/VBAD x2; bortezomib x2	14	7	C/C
10-476	M	47	MM	IIA	PR	262	VCBMP/VBAD x4	36	13	C/C
10-464	F	60	MM	IIIA	PR	375	VCMP/VBAD x6	42	3	C/C
10-515	M	36	NHL	IVA	PD	62	NHL-BMF90 (prephase+AA+BB); IFE x2	16	4	C/C
10-469	F	53	NHL	IE	CR	135	MegaCHOP x4	20	12	C/C
10-544	M	40	NHL	IIB	PR	141	Promace-Cytabom x7; ESHAP x3; CHOP x7	103	5	C/C
10-530	F	57	NHL	IIIA	CR	151	CHOP-R x10; RT 40 Gy	50	5	C/C
10-498	M	62	NHL	IVB	CR	153	HyperCVAD x3 + CHOP-R x4	35	10	C/C
10-527	F	38	NHL	IVB	CR	161	MegaCHOP x4	20	3	C/C
10-549	F	47	NHL	IVA	CR	168	CHOP x6; ESHAP x3	42	6	C/C
10-471	F	58	NHL	IVB	CR	173	CHOP-R x6	30	13	C/C
10-528	M	32	NHL	IVA	CR	178	CHOP-R x6	30	6	C/C
10-550	F	59	NHL	IVA	PR	183	CHOP-R x6; MINE x6	66	5	C/C
10-460	F	34	NHL	IVA	PD	185	CHOP-R x8; FCM x6	64	17	C/C
10-457	M	65	NHL	IVB	CR	193	CHOP-R x6; MINE x6	66	12	C/C
10-534	M	19	NHL	IVA	CR	201	MegaCHOP x4	20	7	C/C
10-459	F	32	NHL	IVA	PR	205	CHOP-R x8, FCM x3	52	12	C/C
10-520	F	53	NHL	IIIB	CR	206	Hyper-CVAD x4	20	5	C/C

10-522	M	53	NHL	IVB	PR	224	Carboplatin/etoposide x6; CHOP-R x8	52	12	C/C
10-489	M	63	NHL	IVB	CR	229	CHOP-R x8; R-MINE x3	58	18	C/C
10-532	M	40	NHL	IVB	PR	246	ABVD x4; ESHAP x4; CHP-R x4	44	4	C/C
10-477	M	50	NHL	IVA	CR	268	CHOP-R x1; HyperCVAD x2	15	7	C/C

[a] M: male; F: female.
[b] ALL: acute lymphoblastic leukemia; AML: acute myeloblastic leukemia; HL: hodgkin lymphoma; MM: multiple myeloma; NHL: non hodgkin lymphoma.
[c] State of disease at stem cell harvest. CR: complete remission; PD: progression of the disease; PR: partial remission.
[d] Baseline platelets ($x10^9$/L) in the patients at initiation of paclitaxel treatment.
[e] Abbreviations of treatments previous to paclitaxel. ABVD: doxorubicin, vinblastine, bleomycin, dacarbazine; CHOP: cyclophosphamide, doxorubicin, vincristine, prednisone; CHOP-R: cyclophosphamide, doxorubicin,vincristine, prednisone, rituximab; CHP-R: cyclophosphamide, vincristine, prednisolone, rituximab; CMOPP: cyclophosphamide, vincristine, procarbazine, mechlorethamin, prednisone; DHAP: dexamethasone, cytarabine, cisplatin; ESHAP: etoposide, methylprednisolone, cytarabine, cisplatin; FCM: fludarabine, cyclophosphamide, mitoxantrone; FLAG: fludarabine, cytarabine; Hyper-CVAD: cyclophosphamide, vincristine, doxorubicin, dexamethasone; IDICE: idarubicin, cytarabine, etoposide; IFE: ifosfamide, etoposide; IFN: interferon; MegaCHOP: cyclophosphamide, doxorubicin, vincristine, prednisone; MINE: mesna, ifosfamide, mitoxantrone, etoposide; Mini-BEAM: carmustine, etoposide, cytarabine, melphalan; NHL-BMF90: Prephase (cyclophosphamide, prednisone), AA course (Ifosfamide, methotrexate, dexamethasone, vincristine, cytarabine, etoposide), BB course (cyclophosphamide, methotrexate, dexamethasone, vincristine, doxorubicien; Pethema LAL-03AR: vincristine, daunorubicin, prednisone, mitoxantrone, cytarabine, dexamethasone, methotrexate, L-asparaginase, mercaptopurine, cyclophosphamide, teniposide; Pethema LAL-93AR: vincristine, daumorubicin, prednisone, L-asparaginase, cyclophosphamide, metothrexate, mitoxantrone; Pethema LAL-RI96: vincristine, daunorubicin, prednisone, L-asparaginase, cyclophosphamide, mercaptopurine, methotrexate, teniposide, cytarabine; Promace-Cytabom: prednisone, methotrexate, cyclophosphamide, doxorubicin, etoposide/cytarabine, bleomycin, vincristine, methotrexate; RT: radiotherapy; VAD: vincristine, doxorubicin, dexamethasone; VBMCP/VBAD: vincristine, carmustine, melphalan, cyclophosphamide, prednisone/vincristine, carmustine, doxorubicin, dexamethasone; VCMP/VBAD: vincristine, melphalan, cyclophosphamide, prednisone/vincristine, carmustine, doxorubicin, dexamethasone.
[f] According to Aksentijevich *et al*. (Cancer biotherapy & radiopharmaceuticals 2002; 17: 399-403). The score obtained for acute leukemia patients is indicated in brackets, however, their type of treatment is not comparable with the other diseases due to the intense dosages with low-medium myelotoxic drugs, for this reason, it is indicated that the score is not applicable (Na).

ARTÍCULO 5: Genome-wide association study identifies ephrin type-A receptors as novel genes influencing paclitaxel-induced sensory peripheral neuropathy

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En preparación.

Resumen: La neuropatía periférica es la toxicidad limitante de dosis del paclitaxel, un fármaco quimioterapéutico ampliamente utilizado para tratar tumores sólidos. Esta toxicidad exhibe una gran variabilidad interindividual de origen desconocido. En este estudio hemos utilizado un estudio de asociación del genoma completo (GWAS) para identificar variantes genéticas asociadas con la neuropatía inducida por el uso del paclitaxel. Se incluyeron 144 pacientes caucásicos de origen europeo tratados con una combinación de paclitaxel y carboplatino y para los cuales se recogieron de forma detallada datos clínicos y de neuropatía. La asociación entre los genotipos y la neuropatía sensorial periférica inducida por paclitaxel fue evaluada mediante regresiones de Cox definiendo como evento la dosis acumulada de paclitaxel en el momento de la aparición de la neuropatía grado 2. El *top hit* de nuestro estudio correspondió al rs17348202, *downstream* del receptor 4 de efrina A (*EPHA4*) y encontramos entre los *top*-25 SNPs asociados con la neuropatía el rs301927 del gen *EPHA6*. También se observó una asociación para el rs1159057 en el gen *EPHA5* (P= 6.8×10^{-5}). Un meta-análisis del rs7349683 en el gen *EPHA5*, el *top hit* de un GWAS previo que también estudiaba la neuropatía inducida por paclitaxel, y en alto desequilibrio de ligamiento con rs1159057 (r^2= 0.79), resultó en una estimación de la proporción de riesgo (HR) de 1.68 veces mayor para los portadores del alelo variante (95%CI= 1.42-1.99, P= 1.4×10^{-9}). Además, mediante imputación se identificaron SNPs adicionales en el locus *LIMK2* con una asociación significativa a nivel GWAS (P= 6.4×10^{-8}). En conclusión, en este estudio proporcionamos una confirmación independiente de que el rs7349683, en el gen *EPHA5,* es un marcador de la neuropatía inducida por paclitaxel. También se sugiere que polimorfimos en otros genes *EPHA*, así como el locus *LIMK2*, podrían desempeñar un papel importante en el desarrollo de esta toxicidad, pudiendo constituir la base para una farmacoterapia individualiza del paclitaxel.

Aportación personal: LJ Leandro-García participó en el diseño del estudio, en la selección de las muestras, en el análisis de la calidad del genotipado y en el análisis estadístico de asociación. Finalmente, LJ Leandro-García participó activamente en la discusión crítica de los resultados y fue el autor principal en la redacción del trabajo para su publicación.

Title: Genome-wide association study identifies ephrin type-A receptors as novel genes influencing paclitaxel-induced sensory peripheral neuropathy

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ABSTRACT

Purpose: Peripheral neuropathy is the dose-limiting toxicity of paclitaxel, a chemotherapeutic drug widely used to treat solid tumors. This toxicity exhibits great inter-individual variability of unknown origin. This study aims to identify genetic variants associated with paclitaxel-induced neuropathy by means of a whole genome approach.

Experimental Design: A genome-wide association study (GWAS) was performed in 144 white European patients uniformly treated with paclitaxel/ carboplatin and for whom detailed data on neuropathy was available. Associations between genotypes and paclitaxel-induced grade 2 sensory neuropathy were assessed by Cox regression, modeling the cumulative dose of paclitaxel.

Results: The top hit of our study corresponded to rs17348202, downstream of ephrin type-A receptor 4 (*EPHA4*), and among the top-25 SNPs associated with neuropathy, rs301927 was located in *EPHA6*.

Furthermore, evidence of association with risk of neuropathy was observed for rs1159057 in *EPHA5* ($P=6.8x10^{-5}$). Meta-analysis of *EPHA5*-rs7349683, the top marker for paclitaxel-induced neuropathy in a previous GWAS, rs7349683, and in high linkage disequilibrium (r2=0.79) with rs1159057, gave a hazard ratio (HR) estimate of 1.68 (95%CI=1.42-1.99, $P=1.4x10^{-9}$). In addition, imputation in *LIMK2* locus revealed SNPs associated at genome wide significance levels ($P=6.4x10^{-8}$).

Conclusions: This study provides independent confirmation of *EPHA5* rs7349683 as the first marker of paclitaxel-induced neuropathy and suggests that other *EPHA* genes, as well as the *LIMK2* locus, may play an important role in the development of this toxicity. The identified SNPs could form the basis for individualized paclitaxel pharmacotherapy.

INTRODUCTION

Paclitaxel is an antineoplastic drug frequently used as first-line treatment for breast, ovarian, lung and prostate cancers. This molecule, first isolated 40 years ago (Wani et al. 1971), binds the cellular microtubules through the β-tubulin subunit, promoting their stabilization, preventing cell division and finally leading to apoptosis (Schiff et al. 1979; Yvon et al. 1999). Although paclitaxel is an effective treatment for various types of cancers, there are associated toxicities that lead to serious clinical limitations in its use. Peripheral neuropathy is the dose-limiting toxicity of paclitaxel (Jordan et al. 2004; Risinger et al. 2009), with most patients treated with the drug developing this side effect (Quasthoff et al. 2002; Balayssac et al. 2011). Peripheral neuropathy is predominantly sensory and is generally axonal, distal, symmetrical, debilitating and painful (Kuroi et al. 2004; Argyriou et al. 2008). Although the causal mechanisms have not been well defined, it is clear that microtubule mediated axonal transport is affected (Nakata et al. 1999; Theiss et al. 2000; Shemesh et al. 2010). Paclitaxel neurotoxicity is dose-dependent and there are various clinical factors that increase the risk of developing it, such as weekly (versus less frequent) regimens, pre-treatment with neurotoxic agents, *diabetes mellitus*, chronic liver disease, alcoholism, use of anti-retroviral drugs, hypothyroidism, nutritional deficits and a personal history of hereditary or acquired polyneuropathies (Rowinsky et al. 1993; Chaudhry et al. 2003; Mielke et al. 2005; Argyriou et al. 2008). However, despite these factors, there is great inter-individual variability in the neurotoxicity of patients receiving similar amounts of paclitaxel under similar protocols, with some patients suffering serious neuropathies which lead to dose reductions and suspensions. The neuropathy usually takes months to disappear, and in the most severe cases the damage to the peripheral nerves can be irreversible. Therefore, the identification of biomarkers to predict the risk of suffering severe neurotoxicity would be of great clinical utility.

Our group and others have applied candidate genes approaches, focused on the pharmacokinetic and phamacodynamic pathways of paclitaxel, to identify genetic variants associated with this toxicity, in some cases with contradictory results, and which can only explain a relatively small fraction of the variation in neuropathy outcome (Green et al. 2006; Sissung et al. 2006; Leskela et al. 2011; Hertz

et al. 2012; Leandro-Garcia et al. 2012). Recently, the first genome-wide association study (GWAS) focused on paclitaxel neuropathy was published, and identified SNPs in genes not previously studied, such as *EPHA5* and *FGD4* (Baldwin et al. 2012), as putative markers of this toxicity. However, validation of these results and meta-analyses using other genome wide approaches are required to unequivocally identify markers of paclitaxel induced-neuropathy of clinical utility. In this study we performed a GWAS with 144 Caucasian cancer patients homogenously treated with paclitaxel/ carboplatin for whom detailed neuropathy data was collected. We provide independent confirmation of *EPHA5* rs7349683 as the first marker of paclitaxel-induced peripheral neuropathy and suggest common variants in other *EPHA* genes, and at *LIMK2* locus, as potential additional markers of susceptibility to this toxicity.

MATERIALS AND METHODS

Patients and peripheral neuropathy assessment

Blood or saliva samples were collected from 144 cancer patients treated with paclitaxel in one Spanish and two Swedish centers: 48 patients (33%) from Hospital Universitario Fundación Alcorcón (Leskela et al. 2011), 63 (44%) from Karolinska Institutet, and 33 (23%) from Linköping University (Green et al. 2011). Eligible patients were over 18 years of age and had: chemotherapy regimen with paclitaxel 175mg/m^2 and carboplatin AUC 5-6 every 21 days to treat a histologically documented solid neoplasia; life expectancy of ≥12 weeks; Eastern Cooperative Oncology Group performance status of 2 or less; no chemotherapy, hormonal therapy nor radiotherapy within 4 weeks before treatment; contraception for women of childbearing potential; adequate bone marrow, renal and hepatic function; and no personal history neuropathy. Most patients had ovary or lung malignancies (70% and 19%, respectively) and were administered paclitaxel/ carboplatin chemotherapy as first line treatment (see Table 1). All patients were of European origin. The collection of samples was approved by the corresponding Internal Ethical Review Committee and all patients gave written informed consent. Additional details related to the patients, tumors and treatments are summarized in Table 1.

Demographic, tumor and treatment data were collected by medical chart review and stored in an electronic database. To guarantee that neuropathy data was collected in a homogenous manner, the three participating centers designed a common questionnaire based on the National Cancer Institute Common Toxicity Criteria (NCI-CTC) version 2, but also incorporating details on sensory symptoms such as numbness/ paresthesia only in the feet, present in both feet and fingers, and functional disabling numbness/ paresthesia, the cycle number and accumulated dose of paclitaxel. A subset of 71 patients undertook a more extensive sensory and motor paclitaxel-induced neurotoxicity assessment (Green et al. 2011; Leskela et al. 2011). Neuropathy symptoms at baseline, accumulated paclitaxel dose at first neuropathy event, at grade 2 sensory neuropathy and at maximum neuropathy grade were also collected

from all patients. Treatment delays, dose reductions and suspensions were also recorded together with the accumulated paclitaxel dose at the change in treatment (see Table 2).

DNA isolation, genotyping, quality control and SNP imputation

DNA was isolated from peripheral blood using the FlexiGene DNA Kit (Qiagen, Valencia, CA, USA) or an automatic DNA extraction robot (Magnapure, Roche, Mannheim, Germany) and from saliva using the Oragene DNA Self-Collection Kits (DNA Genotek Ottawa, ON, Canada), according to the manufacturer's recommended protocols. The final DNA concentration was quantified by PicoGreen (Invitrogen, Carlsbad, CA, USA).

Genome-wide genotyping was performed using 250 ng of DNA with the Infinium assays BeadChip Human 660WQuad (Illumina, San Diego, CA) which consists of 657,366 markers in a 4-sample array format. The GenomeStudio software package was used to extract whole-genome DNA analysis data from image data files created by the Illumina iScan System. One sample with a call rate <0.95, probably due to poor DNA quality, was excluded; all other samples had call rates >0.99. Non-diploid (e.g. mitochondrial chromosomes), CNV probes and SNPs deemed unreliable by Illumina (Tech Note: Infinium® Genotyping Data Analysis, 2007) were excluded leaving 559,348 SNPs. After excluding SNPs with missing genotypes in more than 95% of samples as well as those with MAF <0.025, 518,577 SNPs were included in the association analysis.

Genotypes were imputed for SNPs not genotyped in *EPHA4*, *EPHA6*, *EPHA5*, *EPHA8*, *LIMK2* and *PAPD7* based on the 1000 Genomes Project (June 2011 release) using IMPUTE (version 2.0). Prior to imputation, study genotypes were filtered and limited to autosomal SNPs with MAF ≥0.01 and HWE P-values ≥0.001. Imputed SNPs with MAF <0.025 and call rate <0.90 were excluded.

Statistical analysis

Associations with risk of paclitaxel neuropathy were tested for clinical factors and SNPs using Cox regression analysis, modeling the cumulative dose of paclitaxel up to the development of grade 2 sensory peripheral neuropathy. Patients with no or minimal adverse reaction (grade 0/1) were censored at total paclitaxel cumulative dose (mg). Associations with SNPs were assessed under an additive genetic model by multivariable analysis including age as covariate, since there was weak evidence that older patients were at increased risk of neuropathy (HR per year=1.02; CI95%= 1.00-1.05; P=0.082). Cox regression analysis were performed using the R statistical environment (version 2.14.0); all other statistical analysis were carried out using PLINK (version 1.07). All SNPs with a nominal P-value ≤ 10^{-4} were further evaluated for potential errors by visualizing genotype cluster plots and comparing the estimated MAF with that reported in the HapMap Project, testing HWE and failure rates. To account for possible differences in the ethnic origin of patients, analyses were repeated adjusting for country of origin, with no substantial differences observed in the results obtained (data not shown). Haplotype analysis was conducted for the

LIMK2 region using genotyped SNPs. Haplotype blocks were identified with Haploview v 4.2 software using HapMap CEU samples by the method described by Gabriel et al. (Gabriel et al. 2002) and haplotypes were imputed using PHASE v2.0 (Stephens et al. 2001). The association of each haplotype with the peripheral neuropathy was assessed using Cox regression, adjusted for age.

RESULTS

The distribution of paclitaxel-induced peripheral sensory neuropathy in the patients is shown in Table 2. The median cumulative paclitaxel dose at which patients developed sensory peripheral neuropathy was of 2046 mg and this toxicity caused dose modifications in 25% of patients. There was no significant difference in the incidence of neuropathy between Spanish and Swedish patients (P=0.91). No association with paclitaxel-induced neuropathy was observed with gender, tumor type, tumor stage, previous chemotherapy nor prior used of neurotoxic drugs (P>0.287).

None of the SNPs genotyped in the array were associated with risk of neuropathy at genome-wide statistical significance (defined as $P<10^{-7}$), but associations with $P<10^{-5}$ were observed for several SNPs (Table 3). The strongest evidence of association was observed for rs17348202 (HR=4.85; 95%CI=2.57-9.13, $P=1.02x10^{-6}$; Table 3 and Fig. 1A), located downstream of *EPHA4*, which encodes an ephrin receptor involved in axonal regeneration and functional recovery following spinal cord injury (Munro et al. 2012). Imputation of additional SNPs in *EPHA4* locus did not suggest any stronger association signals (data not shown). Visualization of the linkage disequilibrium (LD) structure within the 1000 Genomes CEU population showed that there was a small region of LD downstream *EPHA4*, but it did not include the coding region of the gene.

Interestingly, a synonymous SNP in *EPHA5* was the top association hit of the previous paclitaxel-induced neuropathy GWAS (rs7349683, HR=1.63, 95%CI=1.34-1.98, $P=9.6x10^{-7}$) (Baldwin et al. 2012). In this study, two SNPs in complete LD, rs1159057 and rs12507286, located in a ~50 kb LD block that included *EPHA5* gene, were among the 40 top hits from our GWAS (HR=2.01, 95%CI=1.43-2.84, $P=6.84x10^{-5}$; Table 4). SNP imputation revealed rs139491476, having a slightly stronger association signal ($P=3.95x10^{-5}$, Suppl. Fig. 3). The previously reported *EPHA5* marker, rs7349683, in high LD with rs1159057 ($r2=0.79$, $D'=1.00$) has in the present GWAS a P value of $3.33x10^{-4}$ (HR=1.83, 95%CI=1.32-2.55; Fig. 1D; Suppl. Fig. 3). A meta-analysis analysis of results from both GWAS gave a P value of $1.4x10^{-9}$ (HR=1.68, 95%CI=1.42-1.99), thus, reaching GWAS statistical significance.

Given that our and the previous GWAS observed the strongest signals for *EPHA4* and *EPHA5*, respectively, and that the EphA family is involved nerve damage response (Goldshmit et al. 2004; Barrette et al. 2010; Goldshmit et al. 2011; Munro et al. 2012), we examined results for SNPs in these genes more closely. An intronic SNP in *EPHA6*, rs301927, was among the 25 most associated SNPs (HR=2.35, 95%CI=1.57-3.53, $P=3.44x10^{-5}$; Table 3, Fig. 1C). Imputation of further SNPs at this locus revealed 4 SNPs in complete LD with a lower association P-value ($P=2.87x10^{-5}$, Suppl. Fig. 2). Furthermore, we

found that rs209709, 12kb upstream of *EPHA8*, had also a P-value of $1.28x10^{-3}$ (HR=2.20, 95%CI=1.36-3.55; Table 4). Interestingly, imputation revealed an *EPHA8* intronic SNP, rs3754005, with a lower P value ($P=9.83x10^{-4}$) and a missense variant, rs606002 coding for *EPHA8* S457P, not in LD with rs209709 (r2=0.011) and constituting an independent association signal ($P=3.36x10^{-3}$). No other SNPs in *EPHA* genes were detected in relation with paclitaxel-induced neuropathy.

The SNP with second-lowest P value was rs4141404 (HR=2.41; 95%CI=1.66-3.48, $P=3.22\ x10^{-6}$; Table 3 and Fig. 1B), located in the 3'UTR of the *LIMK2* gene. The surrounding LD structure obtained from the 1000 Genomes CEU population revealed a ~500 kb region with r2>0.7 including 8 additional genes (*PLA2G3* and *RNF185* upstream, and *PIK3IP1*, *PATZ1*, *DRG1*, *EIF4NIF1*, *SFI1* and *PISD* downstream, see Suppl. Fig. 1). An intronic SNP in *LIMK2*, rs2413045, but with relatively low LD with rs4141404 (r2=0.58), was also among the top 25 associated SNPs ($P=3.67x10^{-5}$; Table 3). Imputation of SNPs that were not genotyped, suggested a reproducible association with paclitaxel-induced peripheral neuropathy that reached genome-wide statistical significance (Suppl. Fig. 1). The lowest P-value corresponded to two SNPs (rs5749227 and rs5749248) in complete LD, located in *RNF185* and *PIK3IP1* genes (respectively), with a MAF of 0.20 (HR=2.96, 95%CI=2.00-4.38, $P=6.38x10^{-8}$). In addition, two coding variants in high LD with rs4141404 were associated with neuropathy: rs2040533, a missense variant in *PIK3IP1*, and rs2228619, a synonymous variant in *LIMK2*, ($P=8.22x10^{-6}$, both). A haplotype analysis of SNPs at this locus did not reveal stronger associations with neuropathy than the single SNP analysis (data not shown).
The third-strongest evidence of association was observed for rs275456, located downstream of *PAPD7* (HR=2.26; 95%CI=1.60-3.18, $P=3.31x10^{-6}$; Table 3). Another SNP, rs13163920, in low LD with rs275456 (r2=0.45) and located in a LD block that included the *PAPD7* gene, was among the 25 top associated SNPs (Table 3). SNP imputation for the entire *PAPD7* locus did not reveal stronger association signals.

It is interesting to note rs3829306, rs4149023 and rs4149013 among the 25-top associated SNPs. These SNPs are in complete LD and are located on introns, and upstream, of *SLCO1B1*, the gene encoding paclitaxel hepatic up-take transporter OATP1B1 (Table 3). None of the other top-25 SNPs were in or near genes previously connected with paclitaxel.

DISCUSSION

We have performed a genome-wide association study to identify genetic variants associated with paclitaxel-induced peripheral neuropathy. This is the dose-limiting toxicity of this widely-used chemotherapeutic drug, and these polymorphisms might serve as markers to define subsets of patients at high risk of neuropathy which could receive alternative chemotherapeutic regimens. It is important to note that neuropathy can decrease the quality of life of the patients and, through early dose reductions and suspensions, also the efficacy of the treatment. Several candidate gene approaches, carried out by our and other groups, have reported that some genes involved in the pharmacokinetic and pharmacodynamic pathways of paclitaxel could be relevant for the neuropathy risk (Green et al. 2006; Sissung et al. 2006;

Leskela et al. 2011; Hertz et al. 2012; Leandro-Garcia et al. 2012). However, there are conflicting results and a major part of the inter-individual variability in paclitaxel-induced neuropathy remains unexplained. In recent years genome-wide approaches have provided the opportunity to assess common variation in the genome in a hypothesis-free manner. A genome-wide study using lymphoblastoid cell lines highlighted the importance of solute carriers mediating paclitaxel-induced cytotoxicity and apoptosis (Njiaju et al. 2012). In addition, a recently published GWAS of paclitaxel-induced neuropathy, based on the clinical trial CALGB 40101, identified putative novel susceptibility loci (Baldwin et al. 2012), although replication in independent studies is required to confirm these. Here we replicate the association of *EPHA5* with paclitaxel-induced sensory peripheral neuropathy reported by Baldwin et al, and propose additional genetic loci at the *LIMK2* locus and in additional *EPHA* receptors.

The ephrin receptor family, is among the largest subfamilies of tyrosine kinase receptors, with 14 distinct members in mammals (EphNomenclatureCommittee 1997). Ephrin receptors can be divided into EphAs and EphBs based on sequence and binding affinity for ephrin-A and -B ligands. There are nine EphAs in humans that are expressed in almost all tissues during development and most cell types of the adult (Hafner et al. 2004). EphA/ephrin-A signaling plays a critical role during embryonic development regulating cell adhesion, positioning and migration and being crucial for nervous system development, tissue regeneration and tumor progression (Miao et al. 2012). EphA5 knockout mice have shown that this receptor is essential in the initiation of the early phases of synaptogenesis (Akaneya et al. 2010), with EphA5 increasing its expression in response to sciatic nerve lesions (Barrette et al. 2010). EphA4 is directly implicated in the regulation of axonal regeneration (Goldshmit et al. 2004; Goldshmit et al. 2011), astrocyte responsiveness (Puschmann et al. 2010; Parmentier-Batteur et al. 2011) and the regulation of other pathways involved in the repair of neural injury (Munro et al. 2012), as shown by the phenotype in knockout mice. EphA6 knockout mice were found to have behavioral deficits as well as learning and memory impairment, suggesting that EphA6, like other EphAs, is involved in neural circuits underlying aspects of learning and memory (Savelieva et al. 2008). Findings in EphA8 knockout mice suggest that this receptor plays a role in axonal pathfinding during the development of the mammalian nervous system (Park et al. 1997). Taken together, these data highlight the relevant function of EphAs in neurons and in pathways involved in repair of neural injury.

In this study we replicate *EPHA5*-rs7349683 as a marker of paclitaxel-induced neuropathy, with the association reaching genome-wide statistical significance in the meta-analysis ($P=1.4x10^{-9}$) with an estimated HR of 1.68 (95% CI=1.42-1.99). Baldwin et al. also included a small replication set in which rs7349683 was genotyped; inclusion of results from this in the meta-analysis gave a HR estimate of 1.59 (95% CI=1.36-1.87) with a P-value of $1.1x10^{-8}$. SNP rs7349683 is a synonymous variant that could subtly affect the protein´s function, leading to a subclinical decrease in nerve injury repair capacity (Barrette et al. 2010) and thereby increasing the risk of neuropathy after paclitaxel exposure. Approximately 48% and 16% of patients will be heterozygous and homozygous, respectively, for the rare allele of rs7349683, and

have an estimated associated 1.68 and 2.82 higher risk of neuropathy, respectively, than wild type homozygous patients.

Interestingly, rs17348202 and rs301927, our 1st and 23rd top associated hits correspond to two ephrin receptors, *EPHA4* and *EPHA6*, respectively. We searched for other SNPs potentially associated with paclitaxel-induced neuropathy in other EphA receptors and found that two independent SNPs in and near *EPHA8* showed weaker evidence of association with paclitaxel peripheral neuropathy ($P=1.3x10^{-3}$ and $6.3x10^{-3}$, respectively). On the whole, these findings suggest that *EPHA* polymorphisms may play a crucial role for paclitaxel-induced neuropathy and should therefore be studied further.

Among the top-25 most statistically significant associations were two independent SNPs located at *LIMK2* locus (rs4141404 and rs2413045, both in *LIMK2* gene; Table 3), which comprises a large block with high LD containing several genes. Imputation of additional variants in the region suggested that associations with two SNPs in complete LD (rs5749227 and rs5749248, Suppl. Fig.1) reached genome-wide statistical significance. Neither the imputation nor the haplotype analysis could identify the gene most likely involved in the neuropathy, although the function of *LIMK2* makes it a promising candidate. This gene encodes a member of the LIM kinase protein family, which is implicated in the reorganization of the actin cytoskeleton in response to a variety of stimuli and plays a central role in the regulation of cell morphology and motility (Scott et al. 2007). *LIMK2* knockout mice exhibited minimal abnormalities, but the *LIMK-1/2* double knockout mice were more severely impaired in excitatory synaptic function (Meng et al. 2004). In addition, *LIMK2* was overexpressed in response to nerve growth factor-induced neurite extension in PC12 cells, and knockdown of *LIMK2* in PC12 cells and in chick dorsal root ganglion neurons resulted in significantly reduced number of neurite-bearing cells, neurite length and cone extension growth rate (Endo et al. 2007; Matsuura et al. 2007). Furthermore, *LIMK2* expression has been implicated in sensitivity to the microtubule-binding agents vincristine and vinblastine in a neuroblastoma cell line (Po'uha et al. 2010). With respect to other genes in this locus, *PIK3IP* encodes for a negative regulator of PI3K (He et al. 2008) and *PLA2G3* is involved in oxidative stress and associated with Alzheimer's disease (Martinez-Garcia et al. 2010).

It is important to highlight that among the 25-top associated SNPs, rs3829306 is an intronic variant located in *SLCO1B1*, the gene encoding OATP1B1, a paclitaxel hepatic up-take transporter previously studied in paclitaxel candidate gene approaches (Gui et al. 2008; Leskela et al. 2011). Two other SNPs on the array, rs4149023 and rs4149013, located on an intron and upstream of *SLCO1B1*, respectively, were in complete LD with rs3829306; however, neither SNP has been described as functional, and no coding SNPs are known to be in high LD with these. The OATP1B1 amino acid change V174A, caused by rs4149056, is known to decrease the transport of several drugs including docetaxel (Niemi et al. 2011; de Graan et al. 2012). However, this variant is not correlated with rs3829306 ($r2=0.012$). One study found an association between the OATP1B1 V174A variant and increased paclitaxel-induced neuropathy ($P=0.021$) (Kroetz et al. 2010) and in this study we estimated a HR

consistent with this finding (HR=1.41, 95%CI=0.95-2.09, P=0.089). These findings together provide some evidence that variants in *SLCO1B1* could modify paclitaxel plasmatic levels and, as a consequence, neuropathy risk.

As discussed previously, we have independently replicated one association from the previous GWAS, confirming that *EPHA5* is a marker of paclitaxel-induced neuropahty (Kroetz et al. 2010). However, we did not find evidence for association for other top hits from that study, such as *FGD4* and *FZD3*. These discrepancies could be due to differences between the two studies. While Baldwin et al. GWAS is based on 855 breast cancer patients treated with paclitaxel monotherapy at 175 mg/m^2 every 2 weeks, we included mostly ovarian and lung cancer patients treated with paclitaxel 175 mg/m^2 plus carboplatin AUC 5-6 every 3 weeks, a widely used paclitaxel schedule (Table 1). We did not find differences in neuropathy among the different types of tumors included, but breast cancer cases were not considered. In addition, the chemotherapy regimens differ among the studies in the frequency of administration, with more frequent paclitaxel administration being more neurotoxic (e.g. weekly regimens) (Argyriou et al. 2008; Seidman et al. 2008), and in the drugs administered, in our study paclitaxel was combined with carboplatin which, although not neurotoxic, may contribute to differences in neuropathy and in the genes associated with it. Both GWAS included patients with European ancestry and the associations with risk of neuropathy were assessed using Cox regression modeling of the cumulative dose of paclitaxel to the development of grade 2 sensory peripheral neuropathy. It is important to highlight the inaccuracy of neuropathy assessment based exclusively on NCI-CTC grading reported by physicians (Cleeland et al. 2010). We used a common neuropathy criterion in the 3 centers recruiting patients, and half of the patients went through a thorough neurologic examination (Green et al. 2011; Leskela et al. 2011). This fact, together with the complete records of accumulated paclitaxel dose in each chemotherapy cycle, allowed us to assess neuropathy accurately and consistently, taking into account the cumulative nature of paclitaxel-induced neurotoxicity. However, the limited number of samples implies a reduced statistical power, making possible that additional associations went undetected. In addition, the high probability of detecting false positive associations by GWAS, makes it crucial to perform a replication of the results in independent studies. Despite these facts and the differences between the studies, both GWAS found an association for EPHA5-rs7349683, thus, indicating that this is a neuropathy marker valid for the different paclitaxel chemotherapy regimens considered. In addition, the nerve injury repair function of EphA5 suggests that this marker could also be valid for other neurotoxic drugs that depend on repair pathways similar to those of paclitaxel.

In summary, this study confirms *EPHA5* rs7349683 as a marker for paclitaxel-induced neuropathy. It also suggests that common variants in other *EPHA* genes and at *LIMK2* locus, could also play a role in this toxicity. Together, these findings suggest that genes involved in the function and repair of peripheral nerves, not previously studied in candidate gene approaches, could make a substantial contribution to the genetic susceptibility to this toxicity. *EPHA5*-rs7349683 appears to be the first marker

which, when combined with others as they emerge, could be used clinically to classify patients according to their neuropathy risk, an important step towards individualized paclitaxel pharmacotherapy.

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TABLES

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

Characteristics	No.	%
Age at treatment (years)		
Median	63	
IQR[a] (minimum–maximum)	69-57 (34–82)	
Gender		
Male	21	15
Female	123	85
Site of primary tumor		
Ovarian	101	70
Fallopian tube	8	6
Peritoneal	5	3
Lung	27	19
Uterus	2	1
Breast	1	1
Tumor stage		
I	15	10
II	8	6
III	83	58
IV	38	26
Paclitaxel+Carboplatin treatment[b]		
Median number cycles (minimum–maximum)	6 (2-9)	
Patients with previous chemotherapy	9	6
Patients with previous neurotoxic drugs	5	3

[a] Interquartile range (IQR).
[b] Paclitaxel 175mg/m^2 + carboplatin 5-6 AUC every 21 days.

Table 2. Peripheral neuropathy caused by paclitaxel.

Origin	Cumulative paclitaxel dose up to grade 2 sensory neuropathy, median ± SE		Dose reductions due to neuropathy		Dose suspensions due to neuropathy	
	(mg)	(mg/m^2)	N.	%	N.	%
Total (n=144)	2046 ± 211	1225 ± 218	19	13	17	12
Sweden (n=96)	2083 ± 303	1225 ± 135	15	16	12	13
Spain (n=48)	1733 ± 157	1050 ± 84	4	8	5	10

[a] Patients that developed grade 2 neuropathy during paclitaxel/ carboplatin treatment

Table 3. Twenty-five SNPs with strongest evidence of association with grade 2 paclitaxel-induced sensory peripheral neuropathy.

SNP[a]	Chromosome	Gene[b]	Major allele	Minor allele	MAF	HR (95% CI)[c]	*P*-value[c]
rs17348202	2	*EPHA4*	A	G	0.05	4.85 (2.57-9.13)	1.02×10^{-6}
rs4141404	22	***LIMK2*** (***PLA2G3-PISD***)[d, e]	C	A	0.25	2.41 (1.66-3.48)	3.22×10^{-6}
rs275456	5	*PAPD7*	C	A	0.24	2.26 (1.60-3.18)	3.31×10^{-6}
rs1165472	1	***RP11-466L17.1***	A	G	0.30	2.36 (1.64-3.40)	3.65×10^{-6}
rs3181157	12	*CD9*	G	A	0.10	3.22 (1.96-5.29)	4.05×10^{-6}
rs10090117	8	*PSD3/ NAT2*	A	G	0.21	2.38 (1.64-3.44)	4.23×10^{-6}
rs10065203	5	***TRIO***	G	A	0.40	2.51 (1.69-3.71)	4.25×10^{-6}
rs2947253	15	*ATPBD4/ RP11-702M1.1*	*A*	G	0.11	3.38 (2.01-5.68)	4.36×10^{-6}
rs10512385	9	*ACTL7B*	A	G	0.14	2.58 (1.71-3.89)	5.70×10^{-6}
rs12699683	7	*AGMO/ DGKB*	C	A	0.09	3.66 (2.08-6.45)	6.65×10^{-6}
rs501461	9	***GLIS3***	A	C	0.39	0.43 (0.29-0.63)	1.24×10^{-5}
rs6846708	4	***PALLD***	A	G	0.07	3.64 (2.03-6.53)	1.50×10^{-5}
rs2425553	20	***PTPRT***[f]	G	A	0.21	2.33 (1.58-3.44)	1.94×10^{-5}
rs1753097	13	***SGCG***	A	G	0.20	2.18 (1.52-3.11)	1.96×10^{-5}
rs6473187	8	***KIAA0146-PRKD***[g]	A	G	0.06	3.37 (1.92-5.91)	2.17×10^{-5}
rs13163920	5	*PAPD7*	G	A	0.27	2.41 (1.60-3.61)	2.23×10^{-5}
rs10932374	2	***ERBB4***	G	A	0.26	2.25 (1.54-3.28)	2.58×10^{-5}
rs3829306	12	***SLCO1B1***[h]	G	A	0.09	3.10 (1.82-5.26)	2.84×10^{-5}
rs8110536	19	***C19orf21***	A	C	0.17	2.24 (1.53-3.27)	2.98×10^{-5}
rs189372	3	*LPP*	G	A	0.39	2.27 (1.55-3.35)	2.98×10^{-5}
rs7655560	4	*HAND2*	G	A	0.06	2.97 (1.78-4.95)	3.27×10^{-5}
rs9365397	6	***PARK2***	A	G	0.04	4.33 (2.16-8.65)	3.42×10^{-5}
rs301927	3	***EPHA6***	A	G	0.17	2.35 (1.57-3.53)	3.44×10^{-5}

rs2413045	22	***LIMK2***	G	A	0.15	2.36 (1.57-3.56)	3.67×10^{-5}
rs12743802	1	*TYW3/ LHX8*	G	A	0.04	4.06 (2.08-7.91)	3.85×10^{-5}

[a] For pairs of SNPs with $r^2 > 0.6$only the SNP with the lowest P value is shown.
[b] Intergenic SNPs are denoted by the closest flanking annotated gene(s).
[c] Multivariable Cox regression analysis adjusted for age.
[d] Bold indicates that the SNP is located in a gene.
[e] rs4141404 is located in *LIMK2*, but there is a large region of linkage disequilibrium ($r^2>0.7$) that covers several genes, from *PLA2G3* to *PISD*.
[f] rs2425553 is in complete LD with rs2425556 and rs3092292.
[g] rs6473187 in *KIAA0146* is in complete LD with rs2632496 in *KIAA0146*, with rs4873774 in *UBE2V2* and with rs8178108 in *PRKD.*
[h] rs3829306 is in complete LD with rs4149013 and rs4149023, both in *SLCO1B1.*

Table 4. SNPs in EPH type A receptors associated with grade 2 paclitaxel-induced peripheral neuropathy.

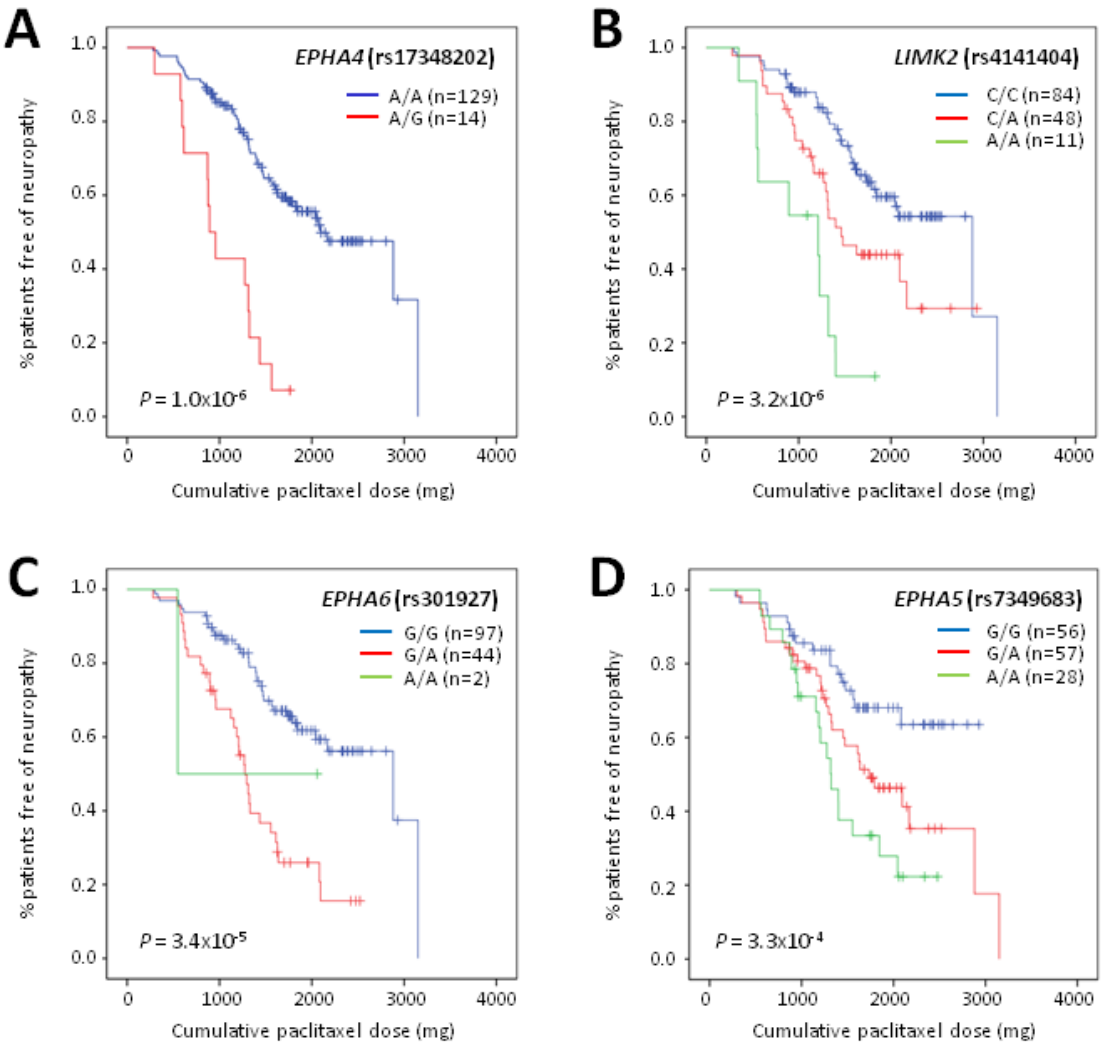
Gene	SNP[a]	Location	Major allele	Minor allele	MAF	HR (95% CI)[b]	P-value[b]
EPHA4	rs17348202	210 kb downstream	G	A	0.05	4.85 (2.57-9.13)	1.02×10^{-6}
EPHA6	rs301927	in gene (intronic)	G	A	0.17	2.35 (1.57-3.53)	3.44×10^{-5}
EPHA5	rs1159057[c]	48 kb downstream, LD covers gene	A	G	0.44	2.01 (1.43-2.84)	6.84×10^{-5}
EPHA8	rs209709	12 kb upstream	G	A	0.17	2.20 (1.36-3.55)	1.28×10^{-3}

[a] Typed SNPs in *EPHA* genes associated with neuropathy with P<0.005. SNPs shown in the Table correspond to the lowest P value in each gene.
[b] Multivariable Cox regression analysis adjusted for age.
[c] rs1159057 is in complete LD with rs12507286.

FIGURES

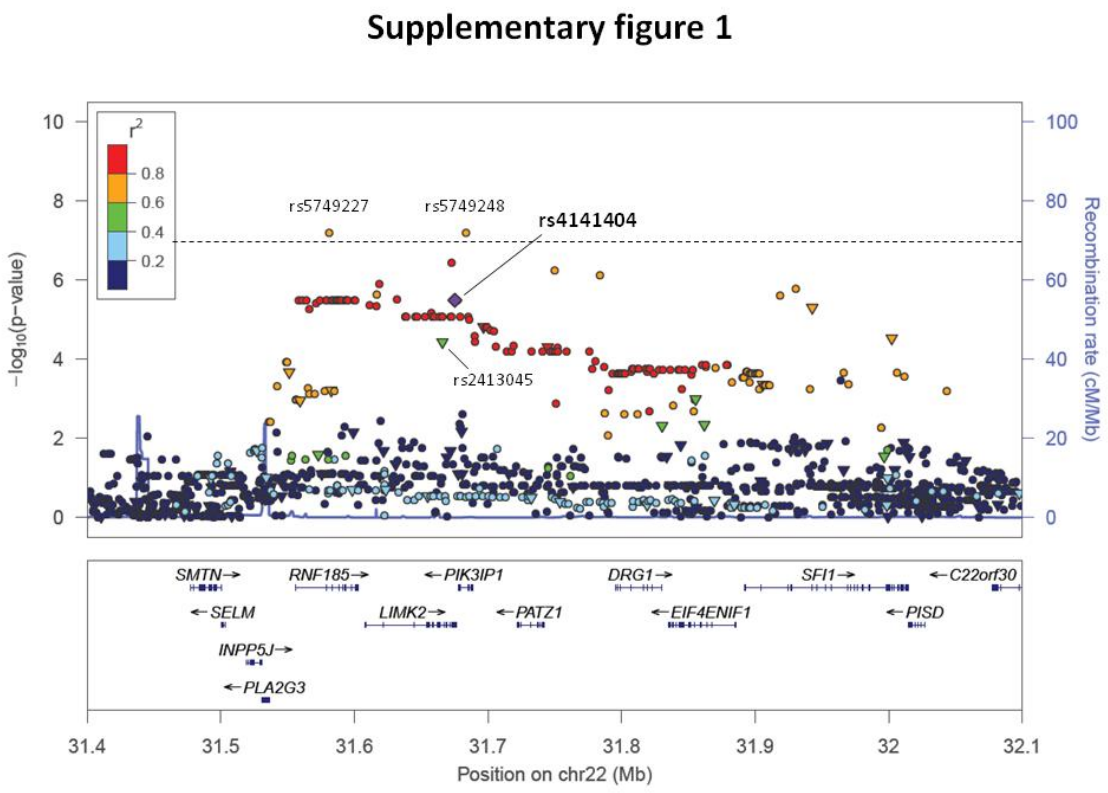
Figure 1. Kaplan–Meier comparisons of cumulative dose of paclitaxel up to the development of grade 2 peripheral sensory neuropathy, by genotype at SNPs in or near *EPHA4*, *LIMK2*, *EPHA6* and *EPHA5*. Patients treated with paclitaxel were grouped according to **A)** *EPHA4*-rs17348202, **B)** *LIMK2*-rs4141404, **C)** *EPHA6*-rs301927 and **D)** *EPHA5*-rs1159057.

Figure 1



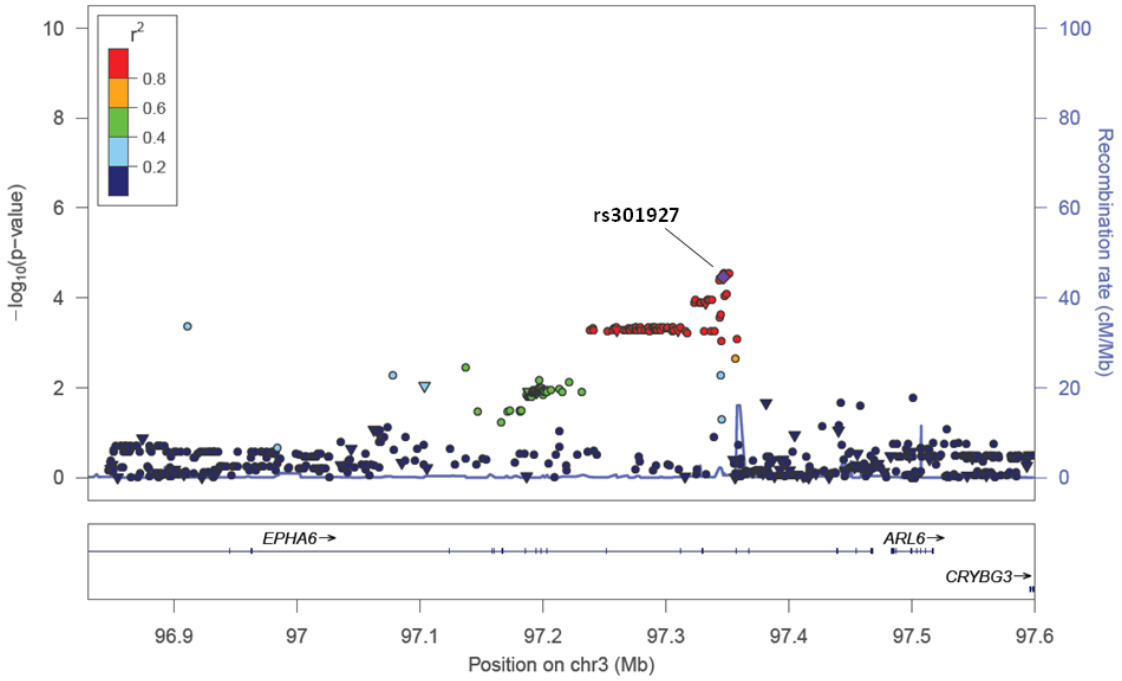
SUPPLEMENTARY FIGURES

Supplementary Figure 1. Regional association plot for the *LIMK2* locus. P-values for genotyped and imputed SNPs surrounding rs4141404. Imputed SNPs are shown as circles, SNPs genotyped on the HumanHap660-Quad BeadChip are triangles, and rs4141404 is a diamond. Recombination rates from HapMap (centimorgans per megabase) are plotted to reflect the local LD structure. Genes are shown in the lower panel of the plot. SNPs are colored to reflect their LD with rs4141404, based on pairwise r2 values from 1000 Genomes Nov 2010 EUR. The figure was generated using LocusZoom (Pruim et al. 2010).



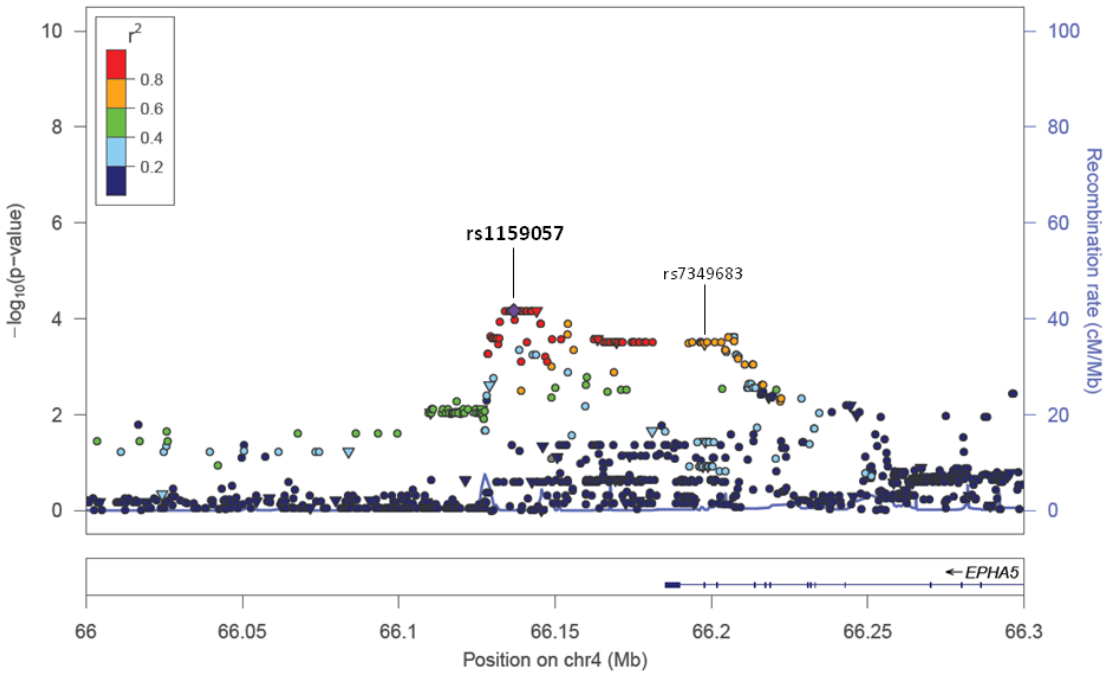
Supplementary Figure 2. Regional association plot for the *EPHA6* locus. P-values for genotyped and imputed SNPs surrounding rs301927. Imputed SNPs are shown as circles, SNPs genotyped on the HumanHap660-Quad BeadChip are triangles, and rs301927 is a diamond. Recombination rates from HapMap (centimorgans per megabase) are plotted to reflect the local LD structure. Genes are shown in the lower panel of the plot. SNPs are colored to reflect their LD with rs301927, based on pairwise r2 values from 1000 Genomes Nov 2010 EUR. The figure was generated using LocusZoom.

Supplementary figure 2



Supplementary Figure 3. Regional association plot for the *EPHA5* locus. P-values for genotyped and imputed SNPs surrounding rs1159057. Imputed SNPs are shown as circles, SNPs genotyped on the HumanHap660-Quad BeadChip are triangles, and rs1159057 is a diamond. Recombination rates from HapMap (centimorgans per megabase) are plotted to reflect the local LD structure. Genes are shown in the lower panel of the plot. SNPs are colored to reflect their LD with rs1159057, based on pairwise r2 values from 1000 Genomes Nov 2010 EUR. The figure was generated using LocusZoom.

Supplementary figure 3



DISCUSIÓN

DISCUSIÓN

I. ESTUDIOS DE GENES CANDIDATOS: β-TUBULINA, DIANA TERAPÉUTICA DEL PACLITAXEL

I.1. Caracterización de la expresión de la diana terapéutica del paclitaxel, los isotipos de β-tubulina, en tejidos humanos no tumorales y tumorales.

La β-tubulina juega un papel crucial en la mitosis y es la diana terapéutica de varios fármacos antineoplásicos ampliamente utilizados en clínica. Sin embargo, la información disponible sobre la expresión de los isotipos de β-tubulina en tejidos tumorales y no tumorales es escasa. En esta tesis hemos desarrollado una cuantificación mediante RT-PCR cuantitativa o qRT-PCR (del inglés *quantitative Real Time-Polymerase Chain Reaction*) para determinar con precisión la expresión de los ARNm de los 8 isotipos humanos de β-tubulina en tejidos no tumorales y tumorales.

Los resultados obtenidos fueron similares a los descritos para otras especies (Cleveland 1990) y para células tumorales (Giannakakou 1997, Hiser 2006, Nicoletti 2001, Ohishi 2007, Verdier-Pinard 2005), a nivel de proteina o de ARNm. Existen algunas discrepancias en los porcentajes de expresión de cada isotipo de β-tubulina (por ejemplo, con el estudio de Nicoletti *et al.*) que podrían deberse a las diferencias existentes entre datos de expresión de ARNm semi-cuantitativos y cuantitativos. Las diferencias con los estudios de Cucchiarelli *et al.* y Dozier *et al*., que también utilizan qRT-PCR (Cucchiarelli 2008, Dozier 2003), podrían deberse a diseños experimentales distintos: diferencias en los oligos específicos, reacciones cruzadas entre isotipos o amplicones de tamaños distintos, entre otras razones.

Con respecto a los patrones de expresión por tejido, el *TUBB* y el *TUBB2C* fueron los isotipos más abundantes en 19 de los 21 tejidos estudiados (cerebro y leucocitos fueron las excepciones). De media, *TUBB* y *TUBB2C* en conjunto representaron más del 75% del contenido total de β-tubulina. Las cantidades de *TUBB2C* excedieron a las del *TUBB* en varios tejidos, con una expresión máxima en testículos, como ya se había sugerido anteriormente (Havercroft 1984). Por otro lado, *TUBB1* tuvo una expresión restringida a tejidos hematopoyéticos, y los isotipos *TUBB2A*, *TUBB2B*, *TUBB3* y *TUBB4* tuvieron niveles de expresión muy altos en cerebro. El gran número y alta expresión de los isotipos expresados en cerebro probablemente refleja la importancia del citoesqueleto para las distintas funciones de las neuronas humanas, si bien algunos de estos isotipos "neuro-específicos" también tuvieron una expresión relevante fuera de cerebro. *TUBB2A* resultó ser el isotipo con mayor expresión extracerebral (ver Figura 2A del Artículo 1). Es interesante resaltar que los genes que codifican para los isotipos IIa y IIb (*TUBB2A* y *TUBB2B*) se encuentran localizados en tándem y que las proteínas β-tubulina IIa y IIb difieren sólo en dos aminoácidos (Figura 3 de la Introducción). Además, al estudiar el genoma del chimpancé común (*Pan troglodytes*) no se encontró la presencia del gen *TUBB2A*, sugiriendo una aparición recientemente en humanos. Las implicaciones funcionales de la duplicación y los cambios de aminoácido

en el isotipo IIa son desconocidas, pero la similitud entre ambos isotipos y su alta expresión nerviosa, sugieren redundancia en este tejido.

Con respecto a la expresión de los isotipos de β-tubulina en tumores, observamos que los isotipos mayoritarios fueron el I (*TUBB*) seguido del IVb (*TUBB2C*). El isotipo I es el predominante en cánceres de ovario, mama y pulmón, indicando que es la principal diana de los tratamientos con taxanos, habituales en estos tumores. El isotipo IVb (*TUBB2C*) tiene una expresión aumentada en líneas de cáncer de mama resistentes a docetaxel (Shalli 2005) y el *knockdown* de este isotipo *in vitro* aumenta la sensibilidad a los vinca alcaloides (Gan 2008). Sin embargo su sobreexpresión o su *knockdown* no parecen cambiar la eficacia del paclitaxel *in vitro* (Blade 1999, Gan 2008). Nosotros observamos una mayor expresión del isotipo IVb en los tumores de mama negativos para el receptor de estrógenos con respecto a aquellos positivos (Figura 4 del Artículo 1). La expresión del isotipo nervioso β-tubulina III (*TUBB3*) se ha asociado *in vitro* con una sensibilidad alterada a los agentes de unión a microtúbulos (Hari 2003, Kamath 2005) y existen crecientes evidencias clínicas que le implican en la resistencia a los taxanos (Ferrandina 2007, Ferrandina 2006, Hasegawa 2003, Mozzetti 2005, Seve 2005, Seve 2007, Seve 2007). La contribución del isotipo III al contenido total de β-tubulina en tumores de mama, ovario y pulmón fue alta (del 18, 16 y 7.5%, respectivamente) apoyando que el isotipo III podría influir en la sensibilidad a los taxanos (Figuras 2C y 3D del Artículo 1). Otro dato interesante fue la disminución de la expresión del isotipo V (*TUBB6*) en todos los tumores estudiados, excepto en los de riñón (Figuras 2C y 3E del Artículo 1 y Figura Suplementaria 1E del Artículo 1). Experimentos *in vitro* en los que se sobre-expresó el isotipo V demostraron que éste confiere cualidades diferentes a los microtúbulos, afectando a su organización, aumentando su fragmentación y resultando en la disrupción del huso mitótico y bloqueo de la proliferación (Bhattacharya 2004). Sin embargo, los tejidos normales estudiados expresan diversas cantidades del isotipo V (Figura 2A del artículo 1).

Los complejos patrones de expresión de los múltiples genes codificantes de los isotipos de β-tubulina, junto con numerosos experimentos *in vitro* (Banerjee 1990, Derry 1997, Panda 1994), sugieren una distinta función para los isotipos, aunque no existen claras evidencias *in vivo* que así lo demuestren. Alternativamente, las duplicaciones génicas podrían haber formado distintos genes que codifican proteínas funcionalmente equivalentes pero que han evolucionado hasta poseer diferentes secuencias reguladoras que confieren una expresión diferencial. Por tanto, todavía son necesarios estudios que sirvan para alcanzar un completo entendimiento del papel biológico de los distintos isotipos de β-tubulina. Este conocimiento es crucial para llevar a cabo estudios racionales con el objetivo de identificar factores que influyan en la resistencia tumoral a agentes de unión a microtúbulos. Por otro lado, las diferencias entre estas proteínas podrían ser explotadas para diseñar agentes de unión a microtúbulos específicos de isotipo, lo que ayudaría a aumentar la eficacia y disminuir las toxicidades asociadas. Por ejemplo, fármacos con una baja afinidad por el isotipo neuronal IVa (*TUBB4*), que no se expresa en tumores, podrían conservar su eficacia terapéutica siendo menos neurotóxicos. Recientemente, se ha demostrado que esta estrategia es factible: la baja afinidad del agente de unión a microtúbulos 2-metoxiestradiol por el

isotipo hematopoyético VI (*TUBB1*) se correlaciona con la ausencia de toxicidad hematológica en pacientes de cáncer tratados con este fármaco (Escuin 2009).

En conjunto, este estudio aporta información clave sobre la expresión normal y tumoral de los isotipos más relevantes de β-tubulina en distintos tejidos humanos. Estos patrones de expresión podrían ayudar al diseño de nuevos fármacos de unión a microtúbulos específicos de isotipo, más eficaces y menos tóxicos.

I.2. Regulación de la expresión de los isotipos de β-tubulina mediante microARNs e implicaciones para la eficacia terapéutica del paclitaxel en cáncer de ovario

El carcinoma de ovario sigue siendo una de las principales causas de muerte por cáncer. La mayoría de los cánceres de ovario son detectados en estadíos avanzados y, aunque ha habido un progreso considerable en el tratamiento de estos tumores, la resistencia a las terapias y la recaída comprometen la supervivencia de las pacientes. Por ello, existe una necesidad de mejorar su tratamiento a través de la identificación de marcadores predictivos de respuesta, incluyendo los microARNs (Adam 2009, Li 2009, Yang 2008, Yang 2008). En esta tesis, encontramos que las 3'UTR de los isotipos de β-tubulina I, IIa y III contenían posibles sitios de unión para la familia miR-200 (Figura Suplementaria 1 del Artículo 2), implicada en el mantenimiento del fenotipo epitelial mediante la represión de los factores de transcripción ZEB1 y ZEB2, y en la progresión tumoral (Gregory 2008). Como la β-tubulina es la diana terapéutica del paclitaxel, estudiamos si la familia miR-200 podría influir en la respuesta y supervivencia de pacientes con cáncer de ovario tratados con paclitaxel y carboplatino.

En este estudio cuantificamos la expresión de los cinco miembros de la familia miR-200 (miR-200b/200c/429 y miR-200a/141) en 72 muestras de cáncer de ovario epitelial (Figura Suplementaria 3 del Artículo 2) y confirmamos una gran variabilidad en su expresión (Hu 2009, Park 2008). La expresión proteica de los isotipos de β-tubulina I, II y III (Figura Suplementaria 2 del Artículo 2) puso de manifiesto diferencias específicas entre isotipos, similares a las descritas previamente (Ohishi 2007). La expresión de los isotipos II y III fue mutuamente excluyente, indicando algún mecanismo común de regulación de su expresión. Con respecto a la β-tubulina III, una sobreexpresión tumoral ha sido asociada a un peor pronóstico (Koh 2009, Seve 2008, Seve 2005), incluyendo carcinomas de ovario (Ferrandina 2006), aunque existe un trabajo con evidencias contrarias en cáncer de ovario de células claras (Aoki 2009). En lo que concierne a la respuesta a taxanos, una alta expresión proteica de β-tubulina III se ha asociado con peor respuesta en cáncer de mama (Hasegawa 2003, Paradiso 2005), pulmón (Rosell 2003, Seve 2005, Seve 2005) y ovario (Umezu 2008). En la serie que hemos estudiado, no detectamos una asociación significativa entre la expresión proteica de la β-tubulina III y la respuesta al tratamiento con paclitaxel-carboplatino, probablemente debido al pequeño número de muestras con alta expresión de esta proteína. Hay evidencias que apoyan que la β-tubulina III es más resistente al efecto de los fármacos de unión a microtúbulos (Cochrane 2009) y de que la falta de expresión tumoral del isotipo II se asocia con

estadios avanzados y una corta supervivencia libre de progresión en carcinoma de ovario (Ohishi 2007). Esto concuerda con la expresión mutuamente excluyente de los isotipos II y III.

En nuestro estudio encontramos que bajos niveles de expresión de los miembros de la familia miR-200 se asociaban de forma significativa con altos niveles de proteína de β-tubulina III (P<0,005 para el miR-141, miR-429, miR-200a y miR-200c y P=0,047 para el miR-200b), sugiriendo que la expresión proteica del isotipo III podría estar regulada por los miembros de esta familia de microARNs. Esta idea es apoyada por recientes estudios *in vitro* en los que la sobre-expresión del miR-200c disminuía la expresión de la proteína β-tubulina III, y aumentaba la sensibilidad a agentes de unión a microtúbulos (Cochrane 2010, Cochrane 2009). Sin embargo, es destacable que no todos los tumores con bajos niveles de expresión de los miembros de la familia miR-200 tenían altos niveles de expresión proteica de la β-tubulina III (Figura 2 del Artículo 2). Esto sugiere mecanismos alternativos de regulación del isotipo III.

No encontramos ninguna relación entre la expresión de los miembros de la familia miR-200 y la expresión proteica de los isotipos de β-tubulina I y II, sugiriendo que los potenciales sitios de unión de la familia miR-200 en estos mensajeros no son funcionales, o que existen otros mecanismos relevantes para la regulación de su expresión, mutaciones en los sitios de unión de los microARNs o procesamiento postranscripcional alternativo de la 3'UTR, procesos que no son infrecuentes en cáncer (Blenkiron 2007, Mayr 2009). Además, para el isotipo de β-tubulina IIa, el único anticuerpo disponible para inmunohistoquímica no discrimina entre este isotipo y el IIb y el potencial sitio de unión para el isotipo IIa se conserva únicamente entre mamíferos. Sin embargo, para el isotipo β-tubulina I, el sitio de unión predicho se conserva entre todos los vertebrados, de manera similar a lo que ocurre con el isotipo β-tubulina III.

Para estudiar el posible papel de los miembros de la familia miR-200 como marcadores de respuesta a los regímenes combinados de paclitaxel y carboplatino, nos centramos exclusivamente en los adenocarcinomas serosos con un estadio FIGO III o IV. Encontramos que las mujeres que carecían de repuesta completa tenían tumores con una expresión del miR-200c significativamente más baja que aquellos tumores de pacientes con respuestas completas (HR=1,43, IC95%=1,02-1,99, P=0.037, Tabla 3 del Artículo 2); además, una expresión mayor del miR-200c también resultó asociada con un menor riesgo a la progresión de la enfermedad o recaída (HR=1,17, IC95%=1,01-1,34, P=0,030, Tabla 3 del Artículo 2). Estos datos sugieren que una baja expresión del miR-200c resulta en una alta expresión proteica de la β-tubulina III y, por tanto, una mayor resistencia a las terapias basadas en paclitaxel. Existen estudios *in vitro* que apoyan esta relación (Cochrane 2010, Cochrane 2009). Si la asociación observada entre la expresión de los miR-200 y la respuesta al tratamiento en primera línea es debida exclusivamente al paclitaxel o la combinación paclitaxel-carboplatino, es algo que se desconoce. En cualquier caso, incluso en el contexto actual, con la aparición de nuevos regímenes que incluyen agentes antiangiogénicos (Cannistra 2007), la existencia de marcadores capaces de identificar pacientes que van a responder a la terapia clásica de paclitaxel-carboplatino sigue siendo muy útil. Este subgrupo de

pacientes respondedoras podría no beneficiarse de las nuevas terapias dirigidas o sufrir reacciones adversas asociadas a su uso, por lo que la terapia clásica sería la más indicada en su caso.

Con respecto al pronóstico de estas pacientes, encontramos que una baja expresión del miR-429 se asociaba tanto a un tiempo libre de progresión de la enfermedad corto como a una supervivencia global corta (P= 0,021 y P=0,041, respectivamente, Figura 3 del Artículo 2). En un modelo de asociación multivariable, ajustado por factores clínico-patológicos relevantes, se observó que la baja expresión del miR-429 mostraba una tendencia a tener un tiempo libre de progresión corto. También se observó dicha tendencia para los miR-200c y miR-141 (Tabla Suplementaria 2 del Artículo 2). En este modelo multivariable no se observó asociación entre el miR-429 y la supervivencia global. Otros estudios han encontrado una asociación significativa entre la expresión del miR-200a y el tiempo libre de progresión de la enfermedad y la supervivencia global (Hu 2009), mientras que otro estudio, con un número bajo de muestras (20 carcinomas serosos de ovario), ha descrito resultados opuestos para los miembros de la familia miR-200 (Nam 2008). Teniendo en cuenta que una baja expresión tumoral de los miembros de la familia miR-200 se ha asociado con progresión y metástasis (Baffa 2009, Gregory 2008, Park 2008), es lógico pensar que sólo por ello la supervivencia global se vería disminuida, independientemente a la respuesta al tratamiento.

Dado que todos los miembros de la familia miR-200 comparten dianas similares, pero existen pequeñas diferencias en los sitios de reconocimiento, proponemos que unos miembros de esta familia podrían ser más importantes para el pronóstico y otros para la respuesta al tratamiento. Además, los niveles de expresión relativos de los miR-200 en las células tumorales podrían estar implicados en la regulación final de los genes diana. Por tanto, una baja expresión tumoral de los componentes de la familia miR-200 podría actuar de forma doble: disminuyendo la respuesta a los agentes de unión a microtúbulos y aumentando el riesgo de metástasis mediante el aumento de genes implicados en la transición de epitelio a mesénquima.

En conclusión, en este trabajo descubrimos que los miembros de la familia miR-200 se asociaban de forma inversa a la expresión de la β-tubulina III en tumores de ovario, sugiriendo una regulación negativa de este gen. Además, nuestros resultados indican un papel de los miembros de esta familia como marcadores predictivos de respuesta al tratamiento con paclitaxel-carboplatino y de pronóstico en carcinoma de ovario. Por tanto, la familia miR-200 podría ser la base para futuras terapias capaces de restituir la expresión de los microARNs de esta familia en las células tumorales. No obstante, estos datos tienen que ser validados en series independientes y en ensayos clínicos prospectivos.

I.3. Polimorfismos reguladores de la expresión de la β-tubulina IIa y su influencia en la neurotoxicidad inducida por el paclitaxel

En este estudio encontramos grandes diferencias interindividuales en la expresión del isotipo de β-tubulina IIa. Este isotipo se expresa en altas cantidades en los microtúbulos neuronales, que son la diana terapéutica del paclitaxel en las neuronas y los últimos responsables de la neurotoxicidad de este fármaco. Por tanto, nuestra hipótesis de partida fue que la variación en la expresión de la β-tubulina IIa podría explicarse por polimorfismos reguladores en la región promotora del gen que contribuirían a las diferencias en neurotoxicidad observadas en los pacientes tratados con paclitaxel. En este respecto, descubrimos que dos polimorfismos en el gen *TUBB2A* en total desequilibrio de ligamiento, -101T>C y -112A>G, tenían una tasa de transcripción mayor y que pacientes portadores de estas variantes tenían menor riesgo a desarrollar neuropatía durante el tratamiento con paclitaxel. Además, apoyando esta asociación encontramos una correlación entre una mayor expresión del gen *TUBB2A* y una menor citotoxicidad del paclitaxel en líneas celulares.

Existen estudios previos que sugieren que la variación genética germinal puede contribuir al riesgo de neurotoxicidad asociada al uso del paclitaxel. En este respecto, estudios en líneas celulares linfoblásticas han estimado que la sensibilidad al paclitaxel tiene una heredabilidad mayor de 0,50, siendo ésta una de las más altas entre 29 fármacos antineoplásicos de uso común testados en un estudio reciente (Peters 2011). Entre los genes que se han asociado previamente a la neuropatía del paclitaxel se encuentran aquellos implicados en la ruta del metabolismo y eliminación de este fármaco: *CYP2C8*, *CYP3A5* y *ABCB1* (Green 2009, Leskela 2011, Sissung 2006). Genes implicados en otras rutas también han sido relacionados con el riesgo a desarrollar neuropatía por paclitaxel. Por ejemplo, dos haplotipos del gen *FANCD2*, que codifica para una proteína de reparación del ADN, se han asociación con los niveles de expresión de este gen y con un mayor riesgo a desarrollar neurotoxicidad (Sucheston 2011), sugiriendo una actividad alterada de la reparación del ADN. Aunque se ha descrito que el paclitaxel podría provocar daño al ADN, como consecuencia del arresto mitótico provocado por este fármaco, el paclitaxel no produce directamente rupturas en el genoma. Por tanto, el mecanismo preciso mediante el que *FANCD2* interfiere con la neuropatía inducida por paclitaxel aún está por dilucidar.

Nuestros resultados constituyen la primera evidencia que apoya que polimorfismos en la diana terapéutica del paclitaxel, la β-tubulina, podrían influir en la toxicidad de este fármaco. Cambios en la composición de los isotipos de β-tubulina se han asociado con diferencias en la respuesta de los tumores al paclitaxel (Ferrandina 2006, Seve 2008, Seve 2005). Específicamente, un aumento en la expresión de la β-tubulina II en carcinomas de cabeza y cuello se ha asociado a una peor respuesta a terapias combinadas que contenían docetaxel (Cullen 2009). También, una expresión aumentada del mensajero del isotipo IIa se ha asociado en líneas celulares con una disminución en la sensibilidad al paclitaxel (Tegze 2012). Estas evidencias están de acuerdo con nuestros resultados, donde encontramos una

correlación inversa entre la expresión del gen *TUBB2A* y la apoptosis inducida por paclitaxel en líneas celulares linfoblásticas (P=0,001, Figura 4 del Artículo 3).

De manera similar, hemos encontrado que pacientes portadores de polimorfismos en el gen *TUBB2A*, que conducen a un aumento en la transcripción del gen, tienen un riesgo disminuido a desarrollar la neurotoxicidad causada por paclitaxel (HR=0,62, IC 95%=0,42-0,93, P=0,021; Figura 3 del Artículo 3). Todos estos datos sugieren que grandes cantidades de β-tubulina II confieren resistencia a la acción de los taxanos. A este respecto, los complejos patrones de expresión de los isotipos de β-tubulina junto con evidencias *in vitro*, sugieren una distinta función y sensibilidad de los isotipos a distintos fármacos (Banerjee 1990, Derry 1997, Panda 1994), lo cual podría explicar por qué una expresión aumentada del gen *TUBB2A* produce una mayor resistencia al paclitaxel.

La gran variabilidad interindividual que hemos observado en la expresión del *TUBB2A* en linfocitos de voluntarios sanos (de hasta 63 veces de diferencia, Figura 1A del Artículo 3) es reflejo de la alta variabilidad genética que hemos encontrado en la región promotora del gen (Figura Suplementaria 2 del Artículo 3). Los ensayos de actividad luciferasa han demostrado que las variantes -101C/-112G del promotor *TUBB2A* son funcionales y afectan a la tasa de transcripción del gen. Aunque el polimorfismo -157G se asoció con una mayor cantidad del mensajero del *TUBB2A* en linfocitos, su presencia no afectaba la actividad luciferasa y no se encontró una asociación entre este polimorfismo y el riesgo a sufrir neurotoxicidad en los pacientes tratados con paclitaxel. Esto sugiere que probablemente esta variante no esté relacionada con los efectos neurotóxicos del paclitaxel, pero hay que tener en cuenta que la frecuencia alélica de este polimorfismo es relativamente baja (0,047), reduciendo el poder estadístico de los estudios de asociación, y que esta variante podría estar en desequilibrio de ligamiento con otro polimorfismo funcional en el promotor del gen. Además, otros polimorfismos en la región promotora del gen *TUBB2A* también podrían estar contribuyendo a la variabilidad observada en su expresión y al riesgo a desarrollar neurotoxicidad debida al paclitaxel.

De forma similar al isotipo IIa, polimorfismos reguladores en otros isotipos de β-tubulina presentes en neuronas, también podrían influir en el riesgo de neuropatía inducida por paclitaxel. En este sentido, encontramos que la variabilidad en la expresión de los isotipos de β-tubulina I y IVb fue muy baja, descartando la existencia de SNPs críticos en las regiones reguladoras de estos genes, pero la expresión de los isotipos IVa, IIb y III, que son fundamentalmente neurona-específicos, no fue detectable en linfocitos de voluntarios sanos y no podemos descartar posibles SNPs reguladores en estos genes.

En conclusión, hemos encontrado una importante variabilidad interindividual en la expresión del gen *TUBB2A* a nivel de ARN mensajero, causada por la mayor tasa de transcripción debida a los polimorfismos -101C/-112G en el promotor del gen. Además, hemos demostrado que la presencia de los polimorfismos reguladores -101C/-112G en pacientes tratados con paclitaxel, se asocia a una protección a desarrollar neuropatía causada por paclitaxel. La correlación inversa entre la apoptosis inducida por paclitaxel y la expresión del *TUBB2A* apoyan este dato. Este es el primer estudio que demuestra una asociación entre la toxicidad del paclitaxel y polimorfismos reguladores en la diana terapéutica del

fármaco, la β-tubulina IIa. De ser confirmados en series independientes, estos polimorfismos podrían ser usados como marcadores del riesgo a desarrollar neurotoxicidad en tratamientos con paclitaxel, sentando la base para una farmacoterapia personalizada del paclitaxel.

I.4. Polimorfismos no-sinónimos en la β-tubulina VI y su relación con la mielosupresión causada por el paclitaxel

En este estudio demostramos que la β-tubulina VI es un isotipo hematológico-específico sujeto a una considerable variabilidad genética, con polimorfismos de cambio de amino ácido frecuentes en la población. Una caracterización de la expresión de la β-tubulina VI en células de linaje hematopoyético reveló una alta expresión de este isotipo no sólo en linfocitos y plaquetas (Escuin 2009, Leandro-Garcia 2010), sino también en la mayoría de las células sanguíneas y en varias neoplasias hematológicas (Figura 1 del Artículo 4). Estos datos junto con la ausencia de expresión de este isotipo en tejidos no-hematopoyéticos (Leandro-Garcia 2010) indican que es un isotipo exclusivamente hematológico y la principal diana que media la mielotoxicidad asociada a los agentes de unión a microtúbulos. Por tanto, la variabilidad genética en este isotipo podría estar contribuyendo a las diferencias interindividuales en el desarrollo de mielosupresión asociada al tratamiento con paclitaxel.

Tradicionalmente se ha considerado que los genes que codifican los isotipos de β-tubulina están altamente conservados (Sullivan 1986); sin embargo, en esta Tesis Doctoral demostramos que la β-tubulina VI se diferencia del resto de isotipos, y posee diversos polimorfismos codificantes comunes en la población. Con anterioridad se ha descrito una asociación entre el polimorfismo p.Q43P del isotipo VI con el riesgo a sufrir hemorragias intracerebrales mediante la modulación de la estructura y función de las plaquetas (Freson 2005, Navarro-Nunez 2007). Además, una variante rara en este isotipo, p.R318W, causa macrotrombocitopenia congénita (Kunishima 2009).

En este estudio identificamos una variante sin sentido, p.Y55X, en heterocigosis en el gen que codifica para el isotipo VI (*TUBB1*), pero los hemogramas del individuo portador no mostraron desviación con respecto a los niveles normales. Esto está de acuerdo con que todas las variantes patogénicas identificadas en los isotipos de β-tubulina hayan sido de cambio de aminoácido (Jaglin 2009, Kunishima 2009, Tischfield 2010) y sugiere que proteínas que se truncan prematuramente y no se integran en el citoesqueleto podrían no ser tan patogénicas como variantes de cambio de aminoácido, que pueden conducir a alteraciones en la dinámica de los microtúbulos (Jaglin 2009, Tischfield 2010). Esto apoyaría la falta de fenotipo patológico para la variante p.Y55X en heterocigosis. El polimorfismo p.R307H está localizado en una región conservada que codifica para el lazo-M de la β-tubulina VI; sin embargo, los algoritmos computacionales PolyPhen y SIFT, predicen un efecto benigno de este cambio de aminoácido. Esto se corroboró al no encontrar efectos significativos de esta variante en estudios de activación y agregación plaquetaria. La variante p.T274M también afecta un residuo conservado en el lazo-M de la β-

tubulina VI, y PolyPhen y SIFT la clasifican como una variante probablemente dañina. Esta variante es relativamente frecuente en la población (3.6% de frecuencia alélica en Caucásicos, Tabla 1 del Artículo 4), sugiriendo que no debe alterar sustancialmente la función de la β-tubulina VI. Con respecto a los agentes de unión a microtúbulos, el residuo 274 está localizado en el sitio de unión para los taxanos (Giannakakou 2000).

En este estudio desarrollamos líneas celulares que expresaban de forma estable la variante de la β-tubulina VI 274M, sola o en combinación con la variante 307H, y demostramos que eran menos sensibles al efecto del paclitaxel en comparación con líneas celulares transfectadas con el vector vacío, la proteína silvestre o la proteína con las variantes 43P o 307H (Figura 3 del Artículo 4). Los isotipos humanos de β-tubulina son esencialmente invariables en el sitio de unión del paclitaxel. En particular, el residuo 274 está conservado en todas las β-tubulinas de vertebrados y en todos sus isotipos (Torin Huzil 2006). Por tanto, resulta interesante que el residuo 274 se localice en una región esencial para la unión del paclitaxel en la β-tubulina. Además, líneas celulares con una mutación adquirida en este mismo residuo en el isotipo I (p.T274I) muestran resistencia a paclitaxel, docetaxel y epotilona A (Giannakakou 2000, Huzil 2007). Estos datos sugieren que la treonina 274 es fundamental para la unión de los taxanos y que variantes en este residuo podrían afectar a la unión del fármaco. Por tanto, pacientes portadores de la variante p.T274M podrían ser más resistentes a los efectos mielosupresores de los taxanos.

Para investigar de forma directa el efecto de los polimorfismos de la β-tubulina VI en la toxicidad hematológica de los taxanos, estudiamos una serie de pacientes tratados con un régimen de paclitaxel de muy alta mielotoxicidad, utilizado para la movilización de células progenitoras hematopoyéticas. Utilizamos una serie reclutada entre 1999 y 2008 de 49 pacientes con neoplasias hematológicas que siguieron un tratamiento de movilización de células progenitoras hematopoyéticas con paclitaxel 170 mg/m^2 i.v. por infusión continua de 24 horas, seguido por 8 µg/kg s.c. de G-CSF de forma diaria (Fernandez 2008). Con este protocolo altamente mielosupresor se dieron leucopenias y trombopenias de grados 3-4 en un 55 y 43% de los pacientes, respectivamente. Aunque sólo dos pacientes fueron portadores de la variante 274M en la β-tubulina VI, éstos desarrollaron grados de trombocitopenia significativamente más bajos que el resto de pacientes tratados (P=0,031; Figura 4 del Artículo 4), indicando que el polimorfismo p.T274M se asocia a protección frente a la trombocitopenia causada por el paclitaxel. Es importante destacar que los portadores del polimorfismo p.T274M tenían características similares al resto de pacientes en términos de edad, estadio de la enfermedad, recuento basal de plaquetas y *score* quimioterapéutico (Tabla Suplementaria 3 del Artículo 4). Respecto al tipo de neoplasias, ambos portadores eran pacientes de mieloma que habían tenido tratamientos quimioterapéuticos anteriores con un importante efecto sobre la producción de plaquetas. Con respecto al intervalo de tiempo transcurrido desde el último tratamiento de quimioterapia hasta el tratamiento para la movilización de progenitores hematopoyéticos, los portadores del polimorfismo se encontraron entre aquellos pacientes en los que el intervalo había sido más corto. No se encontraron diferencias significativas del cambio p.T274M para la leucopenia, pero éstos pacientes fueron tratados con G-CSF

para estimular la producción de neutrófilos, lo cual constituye un factor de confusión muy importante. El efecto del polimorfismo p.T274M en la plaquetopenia inducida por paclitaxel está apoyado por el papel crítico que juega la β-tubulina VI en plaquetas, como demuestran los ratones *knock-out* para la β-tubulina VI (Italiano 2003) y las patologías asociadas a los pacientes con mutaciones en este gen (Kunishima 2009). Aunque en estos estudios no se ha descrito ningún efecto patogénico sobre los neutrófilos, hemos detectado una alta expresión de la β-tubulina VI en estas células (Figura 1A del Artículo 3). Ello, sumado al hecho de que las células que expresan mayoritariamente el isotipo VI son resistentes al agente de unión a microtúbulos 2-metoxiestradiol (Escuin 2009) y que este fármaco no causa mielosupresión (Sweeney 2005), apoya un papel relevante de este isotipo en neutrófilos y, por lo tanto, en la neutropenia inducida por paclitaxel. La similitud estructural del paclitaxel y del docetaxel, que comparten sitio de unión en la β-tubulina con las epitolonas, y el hecho de que las líneas celulares que expresan una β-tubulina I con la variante p.T274I sean resistentes a todos estos agentes (Giannakakou 2000), son suficientes evidencias para emprender otros estudios que investiguen el papel del polimorfismo p.T274M de la β-tubulina VI en la toxicidad hematológica asociada a estos otros fármacos. Se estima que aproximadamente un 7% de los individuos caucásicos son portadores de esta variante y podrían mostrar diferencias en mielosupresión inducida por taxanos.

En resumen, este es el primer estudio que demuestra que la β-tubulina VI es un isotipo específico de tejido hematológico, sujeto a una variabilidad genética y de expresión relevantes. Asimismo, hemos mostrado que el efecto del paclitaxel en la estabilización de los microtúbulos es menor para la β-tubulina VI con el cambio p.T274M. Además, aunque es necesaria una validación en series independientes de pacientes, este trabajo sugiere que este polimorfismo podría constituir un marcador de toxicidad hematológica causada por paclitaxel.

II. ESTUDIO DE GENOMA COMPLETO: GWAS

II.1. Identificación de receptores de efrina tipo A como importantes genes asociados a la neuropatía periférica sensorial del paclitaxel mediante un estudio de asociación del genoma completo

Hemos llevado a cabo un estudio de asociación del genoma completo o GWAS para identificar variantes genéticas asociadas a la neuropatía periférica sensorial inducida por paclitaxel, la toxicidad limitante de dosis de este fármaco. La neuropatía puede disminuir tanto la calidad de vida de los pacientes como la eficacia del tratamiento, en este último caso debido a reducciones de dosis y suspensiones del tratamiento. Estudios previos de genes candidatos, llevados a cabo por nuestro grupo y otros, han puesto de manifiesto que genes involucrados en las vías de farmacocinética y farmacodinámica del paclitaxel podrían ser importantes para el desarrollo de la neuropatía (Green 2006, Hertz 2012, Leandro-Garcia 2012, Leskela 2011, Sissung 2006). Sin embargo, existen resultados

contradictorios y una parte importante de la variabilidad interindividual en la neuropatía inducida por paclitaxel sigue siendo inexplicable. En los últimos años, los GWAS han proporcionado la oportunidad de estudiar la variación genética común en el genoma de forma libre de hipótesis previas. Por ejemplo, un GWAS en líneas celulares linfoblastoides ha puesto de manifiesto la importancia de los transportadores de solutos para la apoptosis y la citotoxicidad inducidas por paclitaxel (Njiaju 2012). Además, otro GWAS basado en el ensayo clínico CALGB 40101, ha estudiado la neuropatía inducida por paclitaxel y ha propuesto nuevos *loci* asociados a ella (Baldwin 2012), aunque necesitan ser confirmados en estudios independientes. En nuestro estudio validamos el *top hit* identificado por Baldwin *et al.* (Baldwin 2012), en el receptor 5 de efrina tipo A (*EPHA5*), y proporcionamos nuevos *loci* en el locus *LIMK2* y en otros *EPHA* asociados al riesgo de neuropatía causada por paclitaxel.

La familia de receptores de efrina está entre las subfamilias más grandes de receptores tirosina quinasa (EphNomenclatureCommittee 1997). Estos receptores pueden dividirse en EphAs y EphBs basándose en la similitud de secuencia y la afinidad por los ligandos efrina tipo A o B. Existen nueve EphAs en humanos que se expresan en casi todos los tejidos durante el desarrollo y en la mayoría de tipos celulares del individuo adulto (Hafner 2004). La señalización EphA/ efrina-A juega un papel fundamental durante el desarrollo embrionario regulando la adhesión, posicionamiento y migración celular, siendo crucial para el desarrollo del sistema nervioso, regeneración tisular y progresión tumoral (Miao 2012). Los ratones *knockout* para el gen *EPHA5* han demostrado que este receptor es esencial en las primeras fases de la sinaptogénesis (Akaneya 2010) y que aumenta su expresión en respuesta a lesiones del nervio ciático (Barrette 2010). EphA4 está directamente implicado en la regulación de la regeneración axonal (Goldshmit 2004, Goldshmit 2011), capacidad de respuesta de los astrocitos a distintos estímulos (Parmentier-Batteur 2011, Puschmann 2010) y regulación de otras vías implicadas en la reparación de lesiones neurales (Munro 2012), como demuestra el fenotipo de los ratones *knockout*. Ratones *knockout* para *EPHA6* sufren un déficit conductual con problemas de aprendizaje y memoria, sugiriendo que EphA6, al igual que otros receptores EphA, participa en circuitos neuronales subyacentes al aprendizaje y la memoria (Savelieva 2008). Los ratones *knockout* para el *EPHA8* sugieren que este receptor juega un papel en el crecimiento axonal durante el desarrollo del sistema nervioso de mamíferos (Park 1997). En conjunto, estos datos ponen de manifiesto el crítico papel que tienen los receptores EphAs en las neuronas y en las vías implicadas en la reparación de lesiones neurales.

En nuestro estudio replicamos el *EPHA5*-rs7349683 como marcador de la neuropatía inducida por el paclitaxel, con una asociación que alcanza la significación GWAS en el meta-análisis ($P= 1,4x10^{-9}$) con un riesgo asociado de 1,68 (IC95%= 1,42-1,99). Baldwin *et al.* también incluyeron en su estudio una pequeña serie de replicación en la que el rs7349683 fue genotipado (Baldwin 2012); la inclusión de estos de datos en el meta-análisis resulta en un HR de 1,59 (IC95%= 1,36-1,87) con un P valor de $1.1x10^{-8}$. El SNP rs7349683 es una variante sinónima que podría afectar ligeramente su actividad, conduciendo a una disminución en la reparación de lesiones nerviosas y aumentando el riesgo de desarrollar neuropatía por la exposición al paclitaxel. Aproximadamente un 48% de los pacientes serán heterocigotos y un 16%

serán homocigotos para la variante del rs7349683, con un riesgo estimado de desarrollar neuropatía 1,68 y 2,82 mayor, respectivamente, que los pacientes homocigotos para el alelo silvestre.

Es interesante destacar que los SNPs rs17348202 y rs301927, la 1ª y 23ª mejor asociación de nuestro GWAS (Tabla 3 del Artículo 5), corresponden dos receptores de efrina, *EPHA4* y *EPHA6*, respectivamente. Por tanto, buscamos otros SNPs potencialmente asociados con la neuropatía en genes *EPHA.* Encontramos que dos SNPs independientes en *EPHA8*: rs209709, 12 kb por encima del gen, y rs606002, que da lugar al cambio de aminácido S457P, mostraron una asociación débil (P= $1,3x10^{-3}$ y $6,3x10^{-3}$, respectivamente). En conjunto, estas evidencias sugieren que polimorfismos en los genes *EPHA* podrían jugar un papel crucial en la neuropatía inducida por paclitaxel y deberían de ser objeto de futuros estudios.

Además, entre la lista de los 25 SNPs con mejor asociación también encontramos dos señales independientes localizadas en el locus del gen *LIMK2* (rs4141404 y rs2413045, ambos en el gen *LIMK2*; Tabla 3 del Artículo 5) localizado en un bloque de alto desequilibrio de ligamiento que contiene varios genes. La imputación de polimorfismos en esta región reveló la existencia de dos SNPs en total desequilibrio de ligamiento (rs5749227 y rs5749248) que alcanzaron niveles de significación GWAS (P=$6,38x10^{-8}$; Figura Suplementaria 1 del Artículo 5). Ni la imputación ni un estudio de haplotipos para este locus han podido identificar inequívocamente el gen implicado en la neuropatía, aunque la función de los genes incluidos en este locus sugiere que *LIMK2* es un candidato prometedor. Este gen codifica para un miembro de la familia de proteínas quinasas LIM, implicados en la reorganización del citoesqueleto de actina en respuesta a una variedad de estímulos, desempeñando un papel central en la regulación de la motilidad y la morfología de la célula (Scott 2007). Los ratones *knockout* para el gen *LIMK2* exhiben anomalías mínimas, pero los ratones doble *knockout LIMK1* y *LIMK2* resultan gravemente perjudicados en la función sináptica excitatoria (Meng 2004). Además, *LIMK2* se sobreexpresa en respuesta al factor de crecimiento inductor de la elongación de las neuritas en células PC12 y la inhibición de la expresión de *LIMK2* en células PC12 y neuronas ganglionares de la raíz dorsal de pollo resultó en un número significativamente menor de células formadoras de neuritas, así como en una menor la longitud y tasa de crecimiento de éstas (Endo 2007, Matsuura 2007). Asimismo, la expresión de *LIMK2* ha sido implicada en la sensibilidad a los agentes de unión a microtúbulos vincristina y vinblastina en una línea celular de neuroblastoma (Po'uha 2010). Con respecto a otros genes en este locus, *PIK3IP* es un regulador negativo de PI3K (He 2008) y *PLA2G3* está involucrado en el estrés oxidativo y se ha asociado con Alzheimer (Martinez-Garcia 2010).

Es importante destacar que entre la lista de los 25 SNPs con mejor asociación encontramos tres variantes en total desequilibrio de ligamiento en *SLCO1B1* (rs3829306, rs4149023 y rs4149013, Tabla 3 del Artículo 5), el gen que codifica para el OATP1B1, un transportador hepático de entrada del paclitaxel incluido previamente en estudios de genes candidatos (Gui 2008, Leskela 2011). Sin embargo, estos SNPs no estaban en desequilibrio de ligamiento con SNPs descritos como funcionales. Por ejemplo, la variante rs4149056, responsable del cambio de aminoácido V174A en el OATP1B1, reduce el transporte

de varios fármacos, incluyendo el docetaxel (de Graan 2012, Niemi 2011), sin embargo, esta variante es independiente del rs3829306 (r^2= 0,012). El estudio de GWAS de Baldwin *et al.* describió una asociación entre la variante OATP1B1 V174A y el riesgo de neuropatía inducida por paclitaxel (P= 0,021) (Baldwin 2012) y nosotros observamos una tendencia en la misma dirección (P= 0,089). Esto proporciona alguna evidencia de que variaciones genéticas en *SLCO1B1* podrían modificar los niveles plasmáticos de paclitaxel y, en consecuencia, el riesgo de neuropatía.

Tal y como discutimos anteriormente, nuestro estudio y el anterior GWAS identifican el gen *EPHA5* como un marcador de neuropatía inducida por paclitaxel (Baldwin 2012). Sin embargo, cuando comparamos nuestros resultados con otras variantes descritas por Baldwin *et al.*, como las encontradas en *FGD4* y *FZD3*, no observamos evidencia de asociación. Estas discrepancias podrían deberse a diferencias entre ambos estudios. Mientras que el estudio de Baldwin *et al.* se basa en 855 pacientes de cáncer de mama tratadas en monoterapia con 175 mg/m^2 de paclitaxel cada 2 semanas, nuestro estudio incluyó principalmente pacientes con cáncer de ovario y pulmón tratados con 175 mg/m^2 de paclitaxel más carboplatino (AUC 5-6) cada 3 semanas. En nuestro estudio no encontramos diferencias en la neuropatía entre los diferentes tipos de tumores incluidos, pero el cáncer de mama no fue considerado. Además, la administración del paclitaxel en regímenes más frecuentes, que son más neurotóxicos (p. ej. regímenes semanales) (Argyriou 2008, Seidman 2008), y la combinación de paclitaxel con carboplatino, aunque este último no produce neuropatía, podrían contribuir a las diferencias observadas en la neuropatía y en los genes que se asocian a ella. Ambos GWAS incluyeron pacientes con ascendencia Europea y la neuropatía se analizó mediante una regresión de Cox modelando la dosis acumulada de paclitaxel al momento del desarrollo de la neuropatía periférica sensorial de grado 2. Es importante destacar que la evaluación de la neuropatía basada exclusivamente en la clasificación realizada por los oncólogos utilizando la escala NCI-CTC, presenta una alta inexactitud (Cleeland 2010). En este sentido, nosotros hemos usado un criterio único de neuropatía en los 3 centros de reclutamiento de pacientes, y a la mitad de éstos se les realizó un examen neurológico extenso (Green 2011, Leskela 2011). Este hecho, junto con la utilización de los datos de dosis acumulada de paclitaxel en cada ciclo de quimioterapia, permitió analizar la neuropatía con precisión. A pesar de estas diferencias, los dos GWAS encontraron una asociación para el *EPHA5* rs7349683, indicando que se trata de un marcador de neuropatía válido para distintos tipos de regímenes de quimioterapia con paclitaxel. Además, la función del *EPHA5* en la reparación de lesiones nerviosas sugiere que este marcador también podría ser válido para otros fármacos neurotóxicos dependientes de vías de reparación similares a las del paclitaxel.

En resumen, hemos identificado el *EPHA5* rs7349683 como un marcador de neuropatía inducida por paclitaxel. Además, variantes comunes en otros genes *EPHA* y en el locus *LIMK2*, también podrían desempeñar un papel importante para esta toxicidad. Esto apoya que genes involucrados en la función y reparación de los nervios periféricos, no estudiados previamente en aproximaciones de genes candidatos, podrían contribuir de forma importante a la susceptibilidad genética a desarrollar esta toxicidad. En

definitiva, el rs7349683 es un marcador que podría utilizarse en la clínica para clasificar a los pacientes según su riesgo de desarrollar neuropatía e individualizar la farmacoterapia del paclitaxel.

CONCLUSIONES

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1. Los isotipos de β-tubulina presentan complejos patrones de expresión en los distintos tejidos humanos pudiendo identificarse isotipos ubicuos (*TUBB*, *TUBB2C* y *TUBB6*), hematológicos (*TUBB1*) y prioritariamente de tejido nervioso (*TUBB2A*, *TUBB2B*, *TUBB3* y *TUBB4*).

2. En los tejidos tumorales existe una expresión alterada de los patrones de expresión de β-tubulina. En concreto, la sobre-expresión de la β-tubulina III es un fenómeno común en diversos tipos de tumores, que en el caso de los tumores de ovario, puede en parte explicarse por una disminución en la expresión de los microARNs de la familia miR-200.

3. La expresión del miR-200c se asocia a una mejor respuesta al tratamiento con paclitaxel-carboplatino en cáncer de ovario avanzado, mientras que el miR-429 se asocia con una mejor supervivencia libre de recaída y supervivencia global. Por tanto, los miembros de la familia de miR-200 podrían representar marcadores tanto de respuesta al tratamiento como de pronóstico en el carcinoma de ovario avanzado.

4. La β-tubulina IIa, codificada por el gen *TUBB2A*, presenta importantes diferencias inter-individuales en su expresión. Los SNPs -101T>C (rs909964) y -112A>G (rs909965) en la región proximal del promotor *TUBB2A* son polimorfismos que confieren una mayor transcripción del gen. Una mayor expresión del *TUBB2A* se asocia en modelos *in vitro* con una menor apoptosis inducida por paclitaxel. Además, estos polimorfismos se asocian con una protección frente a la neuropatía periférica causada por el paclitaxel (HR=0,62, IC95%=0,42-0,93, P=0,021).

5. La β-tubulina VI presenta variantes de cambio de amino ácido comunes en la población. En concreto, el SNP rs35565630 (p.T274M) confiere *in vitro* una mayor resistencia al efecto del paclitaxel en los microtúbulos. Pacientes portadores de esta variante y tratados con paclitaxel desarrollan niveles de trombocitopenia significativamente más bajos que los pacientes homocigotos para la variante silvestre (P=0,031).

6. Estudios de GWAS revelan nuevos *loci* potencialmente asociados al riesgo de desarrollar neuropatía sensorial periférica inducida por paclitaxel, y sugieren un papel relevante para los receptores de efrina tipo A. En concreto, la variante rs7349683 en el gen *EPHA5* es un marcador de riesgo de esta toxicidad (HR=1,68, 95% CI= 1,42-1,99, $P=1,4x10^{-9}$, meta-análisis).

7. Los marcadores identificados en este trabajo de tesis doctoral podrían tener una gran utilidad clínica, ya que podrían ayudar a clasificar a los pacientes según su riesgo de desarrollar mielosupresión, neuropatía o tener una falta de eficacia durante el tratamiento con paclitaxel, contribuyendo por tanto a una farmacoterapia del cáncer más individualizada.

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APÉNDICE:

Otras Publicaciones

1. Lancet Oncol. 2011 Nov;12(12):1143-50.

Single nucleotide polymorphism associations with response and toxic effects in patients with advanced renal-cell carcinoma treated with first-line sunitinib: a multicentre, observational, prospective study.

Garcia-Donas J, Esteban E, Leandro-García LJ, Castellano DE, del Alba AG, Climent MA, Arranz JA, Gallardo E, Puente J, Bellmunt J, Mellado B, Martínez E, Moreno F, Font A, Robledo M, Rodríguez-Antona C.

BACKGROUND: Sunitinib is a tyrosine kinase inhibitor with proven efficacy in renal-cell carcinoma, but some patients do not respond or need dose reductions due to toxicity. Because there are no validated molecular predictors of response or toxicity to sunitinib, we aimed to identify genetic markers predictive of outcome and toxic effects. METHODS: In our observational, prospective study we enrolled previously untreated adults (≥ 18 years) with clear-cell renal-cell carcinoma at 15 institutions in the Spanish Oncology Genitourinary Group in Spain. Patients received sunitinib according to local practice guidelines. We assessed RECIST response, progression-free survival (PFS), overall survival, and toxicity of sunitinib with 16 key polymorphisms in nine genes: VEGFR2 (rs2305948 and rs1870377), VEGFR3 (rs307826, rs448012, and rs307821), PDGFR-α (rs35597368), VEGF-A (rs2010963, rs699947, and rs1570360), IL8 (rs1126647), CYP3A4 (rs2740574), CYP3A5 (rs776746), ABCB1 (rs1045642, rs1128503, and rs2032582), and ABCB2 (rs2231142). We assessed associations with efficacy and toxicity by use of univariable and multivariable analyses (with clinical factors associated with outcomes as covariates). We adjusted for multiplicity using the Bonferroni method; p values of less than 0·0031 before adjustment were deemed to still be significant after adjustment. FINDINGS: We enrolled 101 patients between Oct 10, 2007, and Dec 13, 2010. 95 of these patients were included in toxicity analyses and 89 in the efficacy analyses. Two VEGFR3 missense polymorphisms were associated with reduced PFS with sunitinib on multivariable analysis: rs307826 (hazard ratio [HR] per allele 3·57, 1·75-7·30; p(unadjusted)=0·00049, p(adjusted)=0·0079) and rs307821 (3·31, 1·64-6·68; p(unadjusted)=0·00085, p(adjusted)=0·014). The CYP3A5*1 (rs776746) high metabolizing allele was associated in a multivariable analysis with an increased risk of dose reductions due to toxicity (HR per allele 3·75, 1·67-8·41; p(unadjusted)=0·0014, p(adjusted)=0·022). No other SNPs were associated with sunitinib response or toxicity. INTERPRETATION: Polymorphisms in VEGFR3 and CYP3A5*1 might be able to define a subset of patients with renal-cell carcinoma with decreased sunitinib response and tolerability. If confirmed, these results should promote interventional studies testing alternative therapeutic approaches for patients with such variants.

2. Genes Chromosomes Cancer. 2011 Nov;50(11):922-9.

Detection of the first gross CDC73 germline deletion in an HPT-JT syndrome family.

Cascón A, Huarte-Mendicoa CV, Javier Leandro-García L, Letón R, Suela J, Santana A, Costa MB, Comino-Méndez I, Landa I, Sánchez L, Rodríguez-Antona C, Cigudosa JC, Robledo M.

Hereditary primary hyperparathyroidism (HPT) may develop as a solitary endocrinopathy (FIHP) or as part of multiple endocrine neoplasia Type 1, multiple endocrine neoplasia Type 2A, or hereditary HPT-jaw tumor syndrome. Inactivating germline mutations of the tumor suppressor gene CDC73 account for 14 and 50% of all FIHP and HPT-JT patients, respectively, and have also been found in almost 20% of apparently sporadic parathyroid carcinoma patients. Although more than 60 independent germline mutations have been described, to date no rearrangement affecting the CDC73 locus has been identified. By means of multiplex-PCR we found a large germline deletion affecting the whole gene in a two-generation HPT-JT family. Subsequently array-CGH and specific PCR analysis determined that the mutation spanned ~ 547 kb, and included four additional genes: TROVE2, GLRX2, B3GALT2, and UCHL5. Although no clear mutation-specific phenotype was found associated to the presence of the mutation, further studies are needed to assess whether the loss of the neighboring genes could modify the phenotype of carriers. There was complete absence of nuclear staining in the two HPT-JT-related tumors available. The finding of the first rearrangement affecting the CDC73 gene warrants screening for this tumor suppressor gene inactivation mechanism not only in high-risk CDC73 point mutation-negative HPT-JT families, but also in FIHP patients.

3. Nat Genet. 2011 Jun 19;43(7):663-7.

Exome sequencing identifies MAX mutations as a cause of hereditary pheochromocytoma.

Comino-Méndez I, Gracia-Aznárez FJ, Schiavi F, Landa I, Leandro-García LJ, Letón R, Honrado E, Ramos-Medina R, Caronia D, Pita G, Gómez-Graña A, de Cubas AA, Inglada-Pérez L, Maliszewska A, Taschin E, Bobisse S, Pica G, Loli P, Hernández-Lavado R, Díaz JA, Gómez-Morales M, González-Neira A, Roncador G, Rodríguez-Antona C, Benítez J, Mannelli M, Opocher G, Robledo M, Cascón A.

Hereditary pheochromocytoma (PCC) is often caused by germline mutations in one of nine susceptibility genes described to date, but there are familial cases without mutations in these known genes. We sequenced the exomes of three unrelated individuals with hereditary PCC (cases) and identified mutations in MAX, the MYC associated factor X gene. Absence of MAX protein in the tumors and loss of heterozygosity caused by uniparental disomy supported the involvement of MAX alterations in the disease. A follow-up study of a selected series of 59 cases with PCC identified five additional MAX mutations and suggested an association with malignant outcome and preferential paternal transmission of MAX mutations. The involvement of the MYC-MAX-MXD1 network in the development and progression of neural crest cell tumors is further supported by the lack of functional MAX in rat PCC (PC12) cells and by the amplification of MYCN in neuroblastoma and suggests that loss of MAX function is correlated with metastatic potential.

4. Pharmacogenomics J. 2011 Apr;11(2):121-9.

Polymorphisms in cytochromes P450 2C8 and 3A5 are associated with paclitaxel neurotoxicity.

Leskelä S, Jara C, Leandro-García LJ, Martínez A, García-Donas J, Hernando S, Hurtado A, Vicario JC, Montero-Conde C, Landa I, López-Jiménez E, Cascón A, Milne RL, Robledo M, Rodríguez-Antona C.

Neurotoxicity is one of the most relevant dose-limiting toxicities of the anticancer drug paclitaxel. It exhibits substantial interindividual variability of unknown molecular basis, and represents one of the major challenges for the improvement of paclitaxel therapy. The extensive variability in paclitaxel clearance and metabolism lead us to investigate the association between polymorphisms in paclitaxel elimination pathway and neurotoxicity. We selected 13 relevant polymorphisms in genes encoding paclitaxel metabolizing enzymes (CYP2C8, CYP3A4 and CYP3A5) and transporters (organic anion transporting polypeptide (OATP) 1B1, OATP1B3 and P-glycoprotein) and genotyped them in 118 Spanish cancer patients treated with paclitaxel. After adjusting for age and treatment schedule,CYP2C8 Haplotype C and CYP3A5*3 were associated with protection (hazard ratio (HR) (per allele)=0.55; 95% confidence interval (CI)=0.34-0.89; P=0.014 and HR (per allele)=0.51; 95%CI=0.30-0.86; and P=0.012, respectively) and CYP2C8*3 with increased risk (HR (per allele)=1.72; 95%CI=1.05-2.82; and P=0.032). In each case, the allele causing increased paclitaxel metabolism was associated with increased neurotoxicity, suggesting an important role for metabolism and hydroxylated paclitaxel metabolites. We estimated the HR per paclitaxel-metabolism increasing allele carried across the three polymorphisms to be HR=1.64 (95% CI=1.26-2.14; P=0.0003). The results for P-glycoprotein were inconclusive, and no associations were observed for the other genes studied. The incorporation of this genetic data in treatment selection could help to reduce neurotoxicity events, thereby individualizing paclitaxel pharmacotherapy. These results warrant validation in independent series.

5. Mol Endocrinol. 2010 Dec;24(12):2382-91.

Research resource: Transcriptional profiling reveals different pseudohypoxic signatures in SDHB and VHL-related pheochromocytomas.

López-Jiménez E, Gómez-López G, Leandro-García LJ, Muñoz I, Schiavi F, Montero-Conde C, de Cubas AA, Ramires R, Landa I, Leskelä S, Maliszewska A, Inglada-Pérez L, de la Vega L, Rodríguez-Antona C, Letón R, Bernal C, de Campos JM, Diez-Tascón C, Fraga MF, Boullosa C, Pisano DG, Opocher G, Robledo M, Cascón A.

The six major genes involved in hereditary susceptibility for pheochromocytoma (PCC)/paraganglioma (PGL) (RET, VHL, NF1, SDHB, SDHC, and SDHD) have been recently integrated into the same neuronal apoptotic pathway where mutations in any of these genes lead to cell death. In this model, prolyl hydroxylase 3 (EglN3) abrogation plays a pivotal role, but the molecular mechanisms underlying its inactivation are currently unknown. The aim of the study was to decipher specific alterations associated with the different genetic classes of PCCs/PGLs. With this purpose, 84 genetically characterized tumors were analyzed by means of transcriptional profiling. The analysis revealed a hypoxia-inducible factor (HIF)-related signature common to succinate dehydrogenase (SDH) and von Hippel-Lindau (VHL) tumors, that differentiated them from RET and neurofibromatosis type 1 cases. Both canonical HIF-1α and HIF-2α target genes were overexpressed in the SDH/VHL cluster, suggesting that a global HIF deregulation accounts for this common profile. Nevertheless, when we compared VHL tumors with SDHB cases, which often exhibit a malignant behavior, we found that HIF-1α target genes showed a predominant activation in the VHL PCCs. Expression data from 67 HIF target genes was sufficient to cluster SDHB and VHL tumors into two different groups, demonstrating different pseudo-hypoxic signatures. In addition, VHL-mutated tumors showed an unexpected overexpression of EglN3 mRNA that did not lead to significantly different EglN3 protein levels. These findings pave the way for more specific therapeutic approaches for malignant PCCs/PGLs management based on the patient's genetic alteration.

6. Endocr Relat Cancer. 2010 Jun 1;17(2):317-28.

Allelic variant at -79 (C>T) in CDKN1B (p27Kip1) confers an increased risk of thyroid cancer and alters mRNA levels.

Landa I, Montero-Conde C, Malanga D, De Gisi S, Pita G, Leandro-García LJ, Inglada-Pérez L, Letón R, De Marco C, Rodríguez-Antona C, Viglietto G, Robledo M.

The aim of this study is to assess if common genetic variants located in the CDKN1B locus, coding for the cell cycle inhibitor p27(Kip1), are involved in thyroid cancer susceptibility. Based on the literature and functional predictions, we selected three polymorphisms within the CDKN1B gene (rs2066827 (T326G, V109G), rs34330 (-79C>T) and rs36228499 (-838C>A)) to perform the first case-control study in thyroid cancer involving this locus. We had 649 Spanish patients with sporadic thyroid cancer and 385 healthy representative controls available. Luciferase reporter gene assays, real-time quantitative reverse transcription-PCR and immunoblot experiments were carried out to demonstrate the putative effect of the associated variant. The polymorphism rs34330 (-79C>T) was
identified as a risk factor for developing the follicular variant of papillary thyroid carcinoma (FVPTC), fitting a recessive model (odds ratio=2.12; 95% confidence interval=1.09-4.15; P value=0.023). The risk allele (T) of this single nucleotide polymorphism led to a lower transcription rate in cells transfected with a luciferase reporter driven by the polymorphic p27(Kip1) promoter (P value <0.001). This effect was observed in -79TT genotype control carriers, who showed a tendency towards lower CDKN1B mRNA levels in lymphocytes, as well as at the protein level. This is the first study that identifies CDKN1B as a low-penetrance gene in thyroid cancer, and specifically in FVPTC subtype. We propose a reduced CDKN1B gene transcription depending on the genotype of the -79C>T (rs34330) variant as a novel mechanism underlying p27(Kip1) downregulation.

7. Endocr Relat Cancer. 2010 Jan 29;17(1):7-16.

Overexpression and activation of EGFR and VEGFR2 in medullary thyroid carcinomas is related to metastasis.

Rodríguez-Antona C, Pallares J, Montero-Conde C, Inglada-Pérez L, Castelblanco E, Landa I, Leskelä S, Leandro-García LJ, López-Jiménez E, Letón R, Cascón A, Lerma E, Martin MC, Carralero MC, Mauricio D, Cigudosa JC, Matias-Guiu X, Robledo M.

Therapeutic options for patients with metastatic medullary thyroid carcinoma (MTC) are limited due to lack of effective treatments. Thus, there is a need to thoroughly characterize the pathways of molecular pathogenesis and to identify potential targets for therapy in MTC. Since epidermal growth factor receptor (EGFR) seems to play a crucial role for RET activation, a key feature of MTCs, and several promising EGFR/vascular endothelial growth factor receptor 2 (VEGFR2)-targeted drugs have been developed, the present study was designed to investigate whether these proteins are altered in MTCs. We used a well-characterized series of 153 MTCs to evaluate EGFR activation by sequencing and FISH analysis, and to perform EGFR and VEGFR2 immunohistochemistry. EGFR tyrosine kinase domain mutations were not a feature of MTCs; however, EGFR polysomy and a strong EGFR expression were detected in 15 and 13% of the tumors respectively. Interestingly, EGFR was significantly overexpressed in metastases compared with primary tumors (35 *vs* 9%, P=0.002). We also studied whether specific RET mutations were associated with EGFR status, and found a decrease in EGFR polysomies (P=0.006) and a tendency towards lower EGFR expression for the most aggressive RET mutations (918, 883). Concerning VEGFR2, metastasis showed a higher expression than primary tumors (P=2.8 x 10(-8)). In this first study investigating the relationship between EGFR, RET, and VEGFR2 in a large MTC series, we found an activation of EGFR and VEGFR2 in metastasis, using both independent and matched primary/metastasis samples. This suggests that some MTC patients may benefit from existing anti-EGFR/VEFGR2 therapies, although additional preclinical and clinical evidence is needed.

8. Cell Cycle. 2009 Dec;8(23):3914-24.

The hematopoietic-specific beta1-tubulin is naturally resistant to 2-methoxyestradiol and protects patients from drug-induced myelosuppression.

Escuin D, Burke PA, McMahon-Tobin G, Hembrough T, Wang Y, Alcaraz AA, Leandro-García LJ, Rodríguez-Antona C, Snyder JP, Lavallee TM, Giannakakou P.

Taxanes and other microtubule-targeting drugs (MTDs) represent one of the most effective classes of cancer chemotherapeutics. However, ultimately their utility is limited due to drug-induced myelosuppression. Here we identify 2-Methoxyestradiol (2ME2) as the first MTD able to specifically target tumor cells while sparing the bone marrow from dose-limiting, life-threatening toxicities. Following drug selection with 2ME2, epithelial cancer cells acquired a tubulin mutation at Vbeta236I that impaired the 2ME2-tubulin interaction and rendered cells resistant to 2ME2. We further show that the hematopoietic-specific Hbeta1 tubulin isotype naturally encodes Ibeta236 and is insensitive to 2ME2. Systemic administration of 2ME2 in C57BL6 mice revealed that there was no effect on bone marrow microtubules, in contrast to the taxane or Vinca alkaloid induced toxicities. Similar results were obtained upon drug treatment of human bone marrow and CD34-positive stem/progenitor cells. Herein, we describe the first isotype-targeted chemotherapeutic, setting a new paradigm for the entire class of MTDs, and providing a model that could allow the design of new tubulin inhibitors devoid of myelosuppression. The ability to design a drug with minimal side-effects would significantly augment the chances of clinical success by allowing the use of a truly therapeutic dose rather than the maximally tolerated.

9. Horm Metab Res. 2009 Sep;41(9):672-5.

Rationalization of genetic testing in patients with apparently sporadic pheochromocytoma/ paraganglioma.

Cascón A, López-Jiménez E, Landa I, Leskelä S, Leandro-García LJ, Maliszewska A, Letón R, de la Vega L, García-Barcina MJ, Sanabria C, Alvarez-Escolá C, Rodríguez-Antona C, Robledo M.

Hereditary susceptibility to pheochromocytoma (PCC) and paraganglioma (PGL) represents a very complex genetic scenario. It has been reported that the absence of familial antecedents of the disease does not preclude the existence of a mutation affecting any of the five major susceptibility genes. In fact, 11-24% of apparently sporadic cases (without familial or syndromic antecedents) harbor an unexpected germline mutation, but we do not know what is happening in "truly apparently" sporadic patients (i.e., apparently sporadic cases diagnosed with only one tumor). In the present study, we have analyzed 135 apparently sporadic patients developing a single tumor for the five major susceptibility genes: VHL, RET, SDHB, SDHC, and SDHD. Fourteen percent of cases were found to harbor a germline mutation, and only 2.2% of patients were older than 45 years at onset. By taking into account the tumor location and a threshold age at onset of 45 years, we propose a rational scheme for genetic testing. Analyzing VHL and RET genes would be recommended only in young patients developing a single PCC. On the other hand, genetic testing of SDHD should be done in all patients developing an extra-adrenal tumor before the age of 45, and SDHC could be the responsible gene in cases developing a single head and neck tumor, independently of age. Finally, the analysis of SDHB should always be performed because of its association to malignancy and the low penetrance of mutations affecting this gene.

10. Anal Biochem. 2009 Jun 1;389(1):74-6.

Determination of CYP2D6 gene copy number by multiplex polymerase chain reaction analysis.

Leandro-García LJ, Leskelä S, Montero-Conde C, Landa I, López-Jimenez E, León R, Seeringer A, Kirchheiner J, Cascón A, Robledo M, Rodríguez-Antona C.

Cytochrome P450 2D6 (CYP2D6) copy number variation (CNV) influences the metabolism of 15-25% of clinical drugs. Here we describe a novel multiplex polymerase chain reaction (PCR) analysis method that accurately detects CYP2D6 CNV and CYP2D6*9 allele. It includes the amplification of 2 CYP2D6 and 7 control (AQP1, CYP3A4, MDR1, and SDHB) fluorescent PCR products that are separated on a capillary sequencer and normalized using reference samples. The technique was validated using 27 PCR-restriction fragment length polymorphism (RFLP) pregenotyped samples and further tested in 75 Caucasian samples. The method assigns the correct CYP2D6 copy number, independent of already characterized CYP2D6 single nucleotide polymorphisms (SNPs), and could easily be applied to clinical samples.

11. J Clin Endocrinol Metab. 2009 May;94(5):1701-5.

Genetics of pheochromocytoma and paraganglioma in Spanish patients.

Cascón A, Pita G, Burnichon N, Landa I, López-Jiménez E, Montero-Conde C, Leskelä S, Leandro-García LJ, Letón R, Rodríguez-Antona C, Díaz JA, López-Vidriero E, González-Neira A, Velasco A, Matias-Guiu X, Gimenez-Roqueplo AP, Robledo M.

CONTEXT: The presence of familial history in pheochromocytoma/paraganglioma patients, including syndromic antecedents, leads in the majority of cases to a positive genetic testing for mutations in one of the major susceptibility genes described so far. Furthermore, it has been reported that in the absence of familial antecedents, about 11-24% of patients also carry a mutation in one of these related genes. In these cases, other clinical aspects like bilaterality, multiplicity, location of the tumors, or age at onset can help to recognize the underlying genes involved. OBJECTIVE: The objective of the study was to discuss clinical criteria helpful in the genetic diagnosis, placing special emphasis on apparently sporadic cases. DESIGN: Two hundred thirty-seven nonrelated probands were analyzed for the major susceptibility genes: VHL, RET, SDHB, SDHC, and SDHD. Genetic characterization included both point mutation analysis and gross deletions in the SDH genes performed by multiplex PCR. RESULTS: As expected, all syndromic probands were genetically diagnosed with a mutation affecting either RET or VHL. A total of 79.1% (19 of 24) and 18.4% (31 of 168) of patients presenting with either nonsyndromic familial antecedents or apparently sporadic presentation were found to carry a mutation in one of the susceptibility genes. Finally, we found a Spanish founder effect for two mutations: SDHB c.166_170delCCTCA and SDHD c.129G>A. CONCLUSIONS: Germline mutations are rare in apparently sporadic probands diagnosed after age 40 yr (3.9% in our series) and mainly involve SDHB. Therefore, we recommend prioritizing SDHB genetic testing in patients developing isolated tumors at any age, especially those with extraadrenal location or malignant behavior.

12. Clin Endocrinol (Oxf). 2008 Dec;69(6):906-10.

SDHC mutation in an elderly patient without familial antecedents.

López-Jiménez E, de Campos JM, Kusak EM, Landa I, Leskelä S, Montero-Conde C, Leandro-García LJ, Vallejo LA, Madrigal B, Rodríguez-Antona C, Robledo M, Cascón A.

Head and neck paragangliomas are usually asymptomatic and benign tumours arising mainly from the carotid body and the vagal, tympanic or jugular glomus. The majority of patients develop sporadic masses, and around 30% of cases harbor germline mutations in one of the succinate dehydrogenase genes: SDHB, SDHC or SDHD. In these hereditary cases, the presence of familial antecedents of the disease, multiplicity/bilaterality, young age at onset, and more recently, presence of gastrointestinal stromal tumours, are main factors to be considered. Here we describe a new mutation (c.256-257insTTT) affecting the SDHC gene in a 60-year-old-patient with a single head and neck paraganglioma, and without familial antecedents of the disease. *In silico* splice site analysis showed that this variant created a cryptic splice acceptor site and loss of heterozygosity (LOH) supported the pathogenic role of the mutation. Control population analyses did not detect this variant but revealed a novel SDHC polymorphism that exhibited a frequency of 0.3% (3/1020). This latter finding highlights the importance of assessing the clinical relevance of variants of unknown significance by means of analysing sufficient controls. Despite having found a germline mutation in an older, apparently sporadic patient, we consider that the high costs of analysing all susceptibility genes related to the disease support the recommendation of screening for mutations only in patients fulfilling the above criteria.

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Molecular characterisation of a common SDHB deletion in paraganglioma patients.

Cascón A, Landa I, López-Jiménez E, Díez-Hernández A, Buchta M, Montero-Conde C, Leskelä S, Leandro-García LJ, Letón R, Rodríguez-Antona C, Eng C, Neumann HP, Robledo M.

BACKGROUND: Hereditary susceptibility to familial paraganglioma syndromes is mainly due to mutations in one of six genes, including three of the four genes encoding the subunits of the mitochondrial succinate dehydrogenase complex II. Although prevalence, penetrance and clinical characteristics of patients carrying point mutations affecting the genes encoding succinate dehydrogenase have been well studied, little is known regarding these clinical features in patients with gross deletions. Recently, we found two unrelated Spanish families carrying the previously reported SDHB exon 1 deletion, and suggested that this chromosomal region could be a hotspot deletion area. METHODS: We present the molecular characterisation of this apparently prevalent mutation in three new families, and discuss whether this recurrent mutation is due either to the presence of a founder effect or to a hotspot. RESULTS: The breakpoint analysis showed that all Iberian Peninsular families described harbour the same exon 1 deletion, and that a different breakpoint junction segregates in an affected French pedigree. CONCLUSIONS: After haplotyping the SDHB region, we concluded that the deletion detected in Iberian Peninsular people is probably due to a founder effect. Regarding the clinical characteristics of patients with this alteration, it seems that the presence of gross deletions rather than point mutations is more likely related to abdominal presentations and younger age at onset. Moreover, we found for the first time a patient with neuroblastoma and a germline SDHB deletion, but it seems that this paediatric neoplasia in a pheochromocytoma family is not a key component of this disease.

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Tumoral and Tissue-Specific Expression of the Major Human β-Tubulin Isotypes

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The β-tubulins are microtubule components encoded by a multigene family, which produces slightly different proteins with complex expression patterns. Several widely used anticancer drugs base their activity on β-tubulin binding, microtubule dynamics alteration, and cell division blockage. The expression of these drug targets in tumoral and normal cells could be of crucial importance for therapy outcome, unfortunately, the complex β-tubulin expression patterns have been poorly characterized in human. In this study, we developed a quantitative RT-PCR technique that accurately determines the mRNA expression of the eight human β-tubulin isotypes, encoding class I, IIa, IIb, III, IVa, IVb, V, and VI and applied it to 21 nontumoral tissues and 79 tumor samples belonging to seven cancer types. In the nontumoral tissues, we found that, overall, *TUBB* (I), *TUBB2C* (IVb), and *TUBB6* (V) were ubiquitous, *TUBB1*(VI) was hematopoietic cell-specific, and *TUBB2A* (IIa), *TUBB2B* (IIb), *TUBB3* (III), and *TUBB4* (IVa) had high expression in brain; however, the contribution of the different isotypes to the total β-tubulin content varied for each tissue and had a complex pattern. In tumoral tissues, most isotypes exhibited an altered expression in specific tumor types or related to tumoral characteristics. In general, *TUBB3* showed a great increase in expression while *TUBB6* expression was largely decreased in most tumors. Thus, normal tissues showed a complex β-tubulin isotype distribution, which could contribute to the toxicity profile of the microtubule-binding drugs. In addition, the specific isotypes significantly altered in tumors might represent markers for drug response. © 2010 Wiley-Liss, Inc.

Key Words: β-tubulin, microtubules, isotypes, microtubule-binding drugs

Additional Supporting Information may be found in the online version of this article.

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Introduction

Microtubules are ubiquitous polymers that perform many different functions, being critically involved in mitosis, intracellular transport, asymmetric morphology of neurons, and ciliary and flagellar motility. Microtubules are composed by heterodimers of α- and β-tubulin, which are continuously incorporated and released, rendering microtubules highly dynamic structures [Risinger et al., 2009]. In mammals, there are at least six α- and seven β-tubulin isotypes, encoded by multiple genes that display differential tissue expression [Lopata and Cleveland, 1987; Ludueña, 1998]. The genes are constituted by four exons and three introns, while the nonfunctional pseudogenes lack most or all introns [Berrieman et al., 2004]. The β-tubulin isotypes are well conserved across species and have related amino acid sequences differing primarily within the C-terminal 15–20 amino acids, a region of the protein that is the putative binding site for several microtubule-associated proteins (MAPs) [Sullivan and Cleveland, 1986]. The expression pattern of the different β-tubulin isotypes has been studied in a fragmented manner, mainly using specific antibodies binding to the C-terminal of the different isotypes that cross-reacted between some of the isotypes, in tissues from a variety of mammals (mainly chicken, rat and mouse) and in cell lines of human origin [Cleveland, 1987; Hiser et al., 2006]. Nonetheless, these studies have shown that some β-tubulin isotypes seem constitutive (class I, IVb and maybe V) while other isotypes are restricted to specific tissues (class II, III, and IVa neuronal; class VI haemopoietic) [Sullivan et al., 1986; Wang et al., 1986; Cleveland, 1987; Lopata and Cleveland, 1987; Verdier-Pinard et al., 2005]. However, a comprehensive characterization of the distribution of the β-tubulin isotypes in human tissues has not been performed.

Concerning cancer, several antineoplastic drugs extensively used and effective in a wide range of tumor types base their activity on the alteration of microtubule dynamics (e.g. taxanes and *Vinca* alkaloids). The binding of these drugs to β-tubulin arrests cells in mitosis and finally causes cell death. Class I β-tubulin mutations altering the binding of the drug have been shown to cause acquired resistance in cell lines

[Giannakakou et al., 1997, 2000; Kavallaris et al., 2001; Verrills et al., 2003]; however, in tumors this mechanism does not seem to play a substantial role [Berrieman et al., 2004]. On the other hand, the expression of some β-tubulin isotypes has been shown to change in malignant cells. Although the role of each isotype has not been fully elucidated, several lines of evidence suggest that differential expression of β-tubulin isotypes is involved in anticancer-drug-resistance. More specifically, class III tumoral overexpression has been associated with clinical response to different microtubule-inhibitors [Hasegawa et al., 2003; Mozzetti et al., 2005; Ferrandina et al., 2007, 2006; Seve et al., 2005, 2007a,2007b]. Thus, it is clear that a precise characterization of β-tubulin isotype expression in human normal and tumoral tissues could help us understand the molecular basis of toxicity profiles, drug-sensitivity and resistance in cancer patients treated with microtubule binding drugs and help to customize chemotherapy.

In this study, we present a novel qRT-PCR technique which allows to measure with high accuracy, while avoiding cross-reactions, the human β-tubulin isotypes class I, IIa, IIb, III, IVa, IVb, V, and VI (encoded by *TUBB, TUBB2A, TUBB2B, TUBB3, TUBB4, TUBB2C, TUBB6*, and *TUBB1*, respectively). We used this technique to establish the mRNA expression of these eight human β-tubulin isotypes in 21 nontumoral and 79 tumor samples belonging to seven cancer types, finding complex expression patterns according to the different tissues and tumor type.

Materials and Methods

Human Samples

cDNAs from 20 different nontumoral tissues were obtained from BD Biosciences (human Multiple Tissue cDNA (MTC) panel I and II and human immune system panel). In addition, the study included the following frozen tissues: 10 matched nontumoral/tumoral prostate samples, 4 matched breast nontumoral/tumoral and 23 breast tumor samples, 6 clear cell renal carcinomas, 5 nonsmall cell lung cancer samples, 7 colon adenocarcinomas, 4 ovarian serous carcinomas, and 20 larynx squamous cell carcinomas. Tissue samples were provided by the Tissue Bank Network coordinated by the Molecular Pathology Program of the Spanish National Cancer Centre (CNIO), with the collaboration of the Department of Pathology of several Spanish Hospitals. The main characteristics of the commercial and frozen samples are shown in Table I. All samples were of Caucasian origin and the cancer tissues corresponded to tumors that had not received chemotherapy before the surgery, except for three larynx tumors that received cisplatin.

RNA Isolation and Real-Time Quantitative RT-PCR

RNA was isolated from the frozen tissue using TRI-reagent (Molecular Research Center, Cincinnati, OH). Reverse transcription was performed in a single tube using 1 μg of the total RNA, Superscript II Reverse Transcriptase (Invitrogen, CA) and an oligo dT14 primer following the manufacturer's instructions.

The amounts of the different β-tubulin isotypes mRNA were quantified by real-time PCR with the Sequence Detection System 7900HT (Applied Biosystems, Foster City, CA), using primers designed to be specific for each isotype and probes from the Universal ProbeLibrary Set, Human (Roche). The final concentrations of the primers (shown in Table II) and the probes were 200 nM and 100 nM, respectively, with a final volume of 12 μl. For the amplification reaction, the Universal Master Mix (PE Applied Biosystems) was used and the conditions consisted in an initial step of 95°C for 10 min, followed by 55 cycles of 15 s at 95°C and 1 min at 60°C. For isotypes *TUBB*, *TUBB2B*, and *TUBB2C* the forward primer was located in the 5′UTR; for the rest of the isotypes both forward and reverse primers were located in the coding region. In all cases at least one mismatch provided specificity and avoided cross-reactions. To assess the specificity obtained with these primers and amplification conditions, the 8 β-tubulin isotypes were amplified as described above in five liver and six breast cDNAs, the PCR products of each isotype were pooled together, purified using the PCR Purification Kit (QIAGEN) and sequenced. The sequence obtained for each β-tubulin revealed that only the corresponding isotype was amplified and cross-reactions with other isotypes or pseudogenes was ruled out.

To perform the quantification, standard curves for the different β-tubulin isotypes were generated with serial 1/10 dilutions of the MTC cDNA with the maximal expression in each specific isotype. Normalization was carried out with the internal standard β-glucuronidase (GUS), negative controls were present in all series of PCR reactions and all assays were carried out in triplicates. To obtain comparable amounts of the different β-tubulin isotypes within the same sample, the same fluorescence threshold was used (0.025) for all isotypes' quantification. At this value all reactions were in the initial part of the exponential amplification phase. The cycle at which each sample crossed the threshold (Ct) was recorded for all isotypes and GUS. It was confirmed that the Ct values remained constant from run to run by assessment in two independent experiments. The delta–delta Ct method [Livak and Schmittgen, 2001] was used for the calculation of the different amounts of mRNA: in brief, ΔCt was calculated by subtraction of GUS Ct value to the β-tubulin isotypes Ct values. The ΔCt difference between each sample and a reference sample was calculated (ΔΔCt) and this value was then used in the formula $2^{-\Delta\Delta Ct}$ to calculate the different amounts. For the ΔΔCt calculation to be valid, the amplification efficiencies of the genes under study must be approximately equal [Livak and Schmittgen, 2001]. Using this technique the amplicons of the β-tubulin isotypes and GUS were very similar (less than 130 bp, see Table II) and the PCR primers/conditions were designed using the same criteria, suggesting optimal PCR amplification efficiencies (E) close to 2. This was verified using the equation $E = 10^{[-1/\text{slope}]}$ [Rasmussen, 2001].

Table I. Characteristics of the Human Tissues Used for qRT-PCR

Tissues	Normal/tumoral	Sample type	Number of samples and characteristics
Brain[a]	Normal	cDNA	Pool from 2
Heart	Normal	cDNA	Pool from 3
Kidney	Normal	cDNA	Pool from 5
Lung	Normal	cDNA	Pool from 4
Liver[a]	Normal	cDNA	1
Colon[b]	Normal	cDNA	Pool from 20
Small intestine[c]	Normal	cDNA	Pool from 32
Pancreas	Normal	cDNA	Pool from 15
Testis	Normal	cDNA	Pool from 45
Prostate	Normal	cDNA	Pool from 32
Ovary	Normal	cDNA	Pool from 14
Placenta	Normal	cDNA	Pool from 17
Bone marrow	Normal	cDNA	Pool from 74
Leukocytes	Normal	cDNA	Pool from >100
Spleen	Normal	cDNA	Pool from 3
Lymph node	Normal	cDNA	Pool from 12
Thymus	Normal	cDNA	Pool from 9
Tonsil	Normal	cDNA	Pool from 5
Skeletal muscle	Normal	cDNA	Pool from 8
Fetal liver	Normal	cDNA	Pool from 32 fetuses
Prostate	Normal	Frozen	10 normal matched[d]
Breast	Normal	Frozen	4 normal matched[d]
Renal	Tumoral	Frozen	6 clear cell renal cell carcinomas, grades 2-3
Lung	Tumoral	Frozen	5 non small cell lung cancers
Colon	Tumoral	Frozen	7 adenocarcinomas, T3N0 to T4N2
Prostate	Tumoral	Frozen	10 adenocarcinomas, grades 2–4[d]
Breast	Tumoral	Frozen	27 infiltrating ductal carcinoma, grades 1–3[d]
Ovary	Tumoral	Frozen	4 serous carcinoma, grades 2–3
Larynx	Tumoral	Frozen	20 squamous cell carcinomas, well-poorly differentiated

[a]Whole.
[b]With mucosal lining.
[c]Without mucosal lining.
[d]Matched tumoral/ nontumoral samples.

For normal prostate, both MTC cDNA and 10 frozen samples were available, and the isotype expression patterns were compared. The similar results obtained with these samples indicated that they were comparable (e.g. in all cases 99% of the total β-tubulin consisted on *TUBB2C, TUBB, TUBB2A*, and *TUBB6*, in decreasing abundance order, data not shown).

Statistical Analysis

Data were analyzed using GraphPad InStat version 3.00 for Windows 95 (San Diego, CA). The method of Kolmogorov-Smirnov was used to test for normality, and when it indicated normality, parametric tests were used. Differences were considered significant when *P*-values were less than 0.05.

Results

Quantitative RT-PCR Measurement of the β-Tubulin Isotypes mRNA in Nontumoral Samples: Tissue-Specific Expression of the Human β-Tubulin Isotypes

Different public databases were used to determine the most abundant relevant human β-tubulin isotypes (using expression levels among different tissues as the criteria for selection, e.g. GeneNote http://bioinfo2.weizmann.ac.il/cgi-bin/genenote/GN_search.pl and CGAP http://cgap.nci.nih.gov/). In consequence, eight different isotypes encoded by the genes *TUBB* (class I), *TUBB1* (VI), *TUBB2A* (IIa), *TUBB2B* (IIb), *TUBB2C* (IVb), *TUBB3* (III), *TUBB4* (IVa), and

Table II. Primer Sequences and Labeled Probes Used for Quantitative RT-PCR

Gene name	Protein name	Accession number	Forward primer[a]	Position[b]	Reverse primer	Position[b]	Probe (5′–3′)	Protein length (bp)
TUBB (HM40)	I	NM_178014	atacctgaggcgagcaaaa	92–111	ctgatcacctcccagaacttg	181–201	UPL #64[c]	110
TUBB1 (Hβ1)	VI	NM_030773	ggatgcgtgaaattgtccat	268–287	agtcgatcccgtgttcctc	345–363	UPL #14	96
TUBB2A (Hβ9[c])	IIa	NM_001069	aaatatgtacctcgggccatc	257–278	gttattccggctccactct	366–386	UPL #50	129
TUBB2B (Hβ9)	IIb	NM_178012	aggacggacagacccagac	43–61	ctgatgacctcccaaaacttg	148–168	UPL #79	126
TUBB2C (Hβ2)	IVb	NM_006088	ttgtctacttcctcctgcttcc	61–82	ctgatcacctcccaaaacttg	156–176	UPL #70	116
TUBB3 (Hβ4)	III	NM_006086	gcaactacgtgggcgact	166–183	cgaggcacgtacttgtgaga	231–250	UPL #78	85
TUBB4 (Hβ5)	IVa	NM_006087	ccggacaacttcgtgtttg	366–384	acagcgtccaccagctct	437–454	UPL #61	89
TUBB6	V	NM_032525	aggctacgtgggagactcg	147–165	gccctgggcacatatttct	215–233	UPL #78	87
GUS		NM_000181	gaaaatacgtggttggaggctcatt	1809–1834	ccgagtgaagatccccttttta	1888–1909	FAMccagcactctcgtcggtgactgttca3BQ1	101

[a]Primer sequences are shown 5′–3′.
[b]The position of the oligonucleotides is referred to the first nucleotide of the mRNA sequence of the corresponding Accession number.
[c]Universal Probe Library (Roche).

TUBB6 (V), were selected for the mRNA quantification technique (accession numbers for each isotype are shown in Table II).

The mRNA expression of the selected β-tubulin isotypes was assessed by using the quantitative RT-PCR technique described in Materials and Methods in 21 nontumoral human tissues. As shown in Fig. 1, the tissues with the highest β-tubulin expressions were as follows: thymus for *TUBB*; peripheral blood leukocytes for *TUBB1*; brain for *TUBB2A, TUBB2B, TUBB3*, and *TUBB4* and heart for *TUBB2C* and *TUBB6*. When comparing the expression of each isotype among the tissues it was evident that *TUBB2A*, *TUBB2B*, *TUBB3*, and *TUBB4* were neuronal-specific isotypes showing much lower expression in all other tissues. *TUBB1* expression was hematopoietic cell-specific with high expression in leukocytes, bone marrow, and fetal liver. *TUBB*, *TUBB2C*, and *TUBB6* were ubiquitously distributed, although *TUBB6* was more important in heart and *TUBB2C* in both heart and testis. The total β-tubulin content, taking into account the eight isotypes here studied, was found maximal for brain, followed by heart and testis (data not shown). These results are similar to previous reports and are in accordance to the key importance of microtubules for these tissues functions.

With respect to the percentage that each isotype represented in each of the 21 nontumoral tissues (Fig. 2a), we found that *TUBB* and/or *TUBB2C* were the major isotypes in all tissues except for brain. Ovary, lymph node, thymus and fetal liver had *TUBB* as the major isotype. Testis expressed mainly *TUBB2C* and very little *TUBB* (more than 50-fold difference) and kidney, heart, skeletal muscle, and lung had more than 3-fold greater levels of *TUBB2C* than *TUBB*, and could be considered mainly *TUBB2C* expressors. On the other hand, in brain, the expression of most isotypes was very different from all of the other tissues studied, and neither *TUBB2C* nor *TUBB* were the major β-tubulin isotypes (Fig. 2a, first bar). Leukocytes expression pattern was also atypical with *TUBB1* being the most abundant isotype (Fig. 2a, eighth bar).

With respect to other isotypes, *TUBB2A* was also present at relevant amounts in a large number of tissues. Its highest expression occurred in brain, where it represented 30% of all β-tubulins, followed by pancreas, liver, small intestine, breast, kidney, prostate, and placenta where its expression ranged between 22 and 11%. *TUBB4* was the major brain isotype (46%), and it also had an important contribution in spleen and testis (representing 13 and 10% of the β-tubulins, respectively). *TUBB1* was the major isotype in leukocytes (50% of all β-tubulins) and it was also expressed in hematopoietic tissues such as fetal liver, bone marrow and spleen, (9, 6, and 3%, respectively), while in the rest of the tissues *TUBB1* represented less than 0.8%. Maximal *TUBB6* expression occurred in breast and lung with 11 and 10% of total β-tubulins, respectively.

The clustering of the isotypes according to their tissue specific expression showed that brain had an expression pattern very different from the other tissues (Fig. 2b). The hematopoietic tissues bone marrow, fetal liver, and spleen clustered

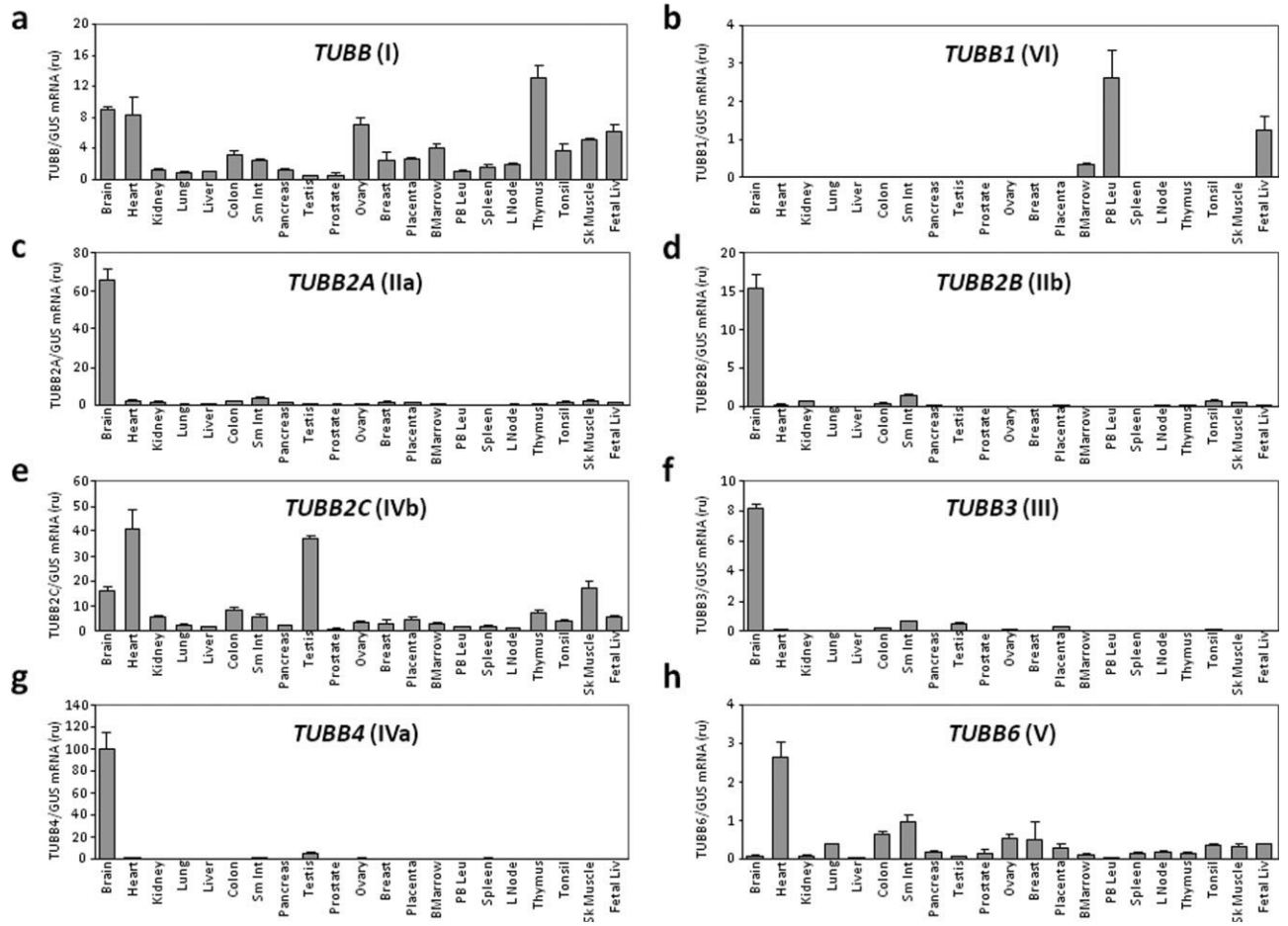

Fig. 1. Expression of eight different human β-tubulin isotypes in human nontumoral tissues. The mRNA content of the β-tubulin isotypes *TUBB, TUBB1, TUBB2A, TUBB2B, TUBB2C, TUBB3, TUBB4*, and *TUBB6* (corresponding to class I, VI, IIa, IIb, IVb, III, IVa, and V isotypes, respectively) was measured by quantitative RT-PCR in 21 normal human tissues, as described in Materials and Methods section. All tissues corresponded to adults except for one fetal sample (fetal liver). The amount of the β-tubulin isotype's mRNA was normalized with the mRNA content of GUS in each sample. The results are referred to the isotype and tissue with the highest β-tubulin expression (*TUBB4* in brain) and represent the mean ± standard deviation.

together, and the same arm contained other tissues related with lymphocyte maturation and accumulation (thymus and lymph nodes); however, ovary, being an unrelated tissue, was also in this arm. The rightmost arm of the cluster contained most of the digestive system organs. With respect to the genes, the ubiquitous *TUBB, TUBB2C*, and *TUBB6* clustered together, as well as the neuronal-specific *TUBB2A, TUBB4, TUBB2B*, and *TUBB3,* whereas the expression pattern of *TUBB1* was the most divergent, similarly to Fig. 1 results.

Human β-Tubulin Isotypes mRNA Expression in Tumoral Samples and Comparison With Nontumoral Tissues

The mRNA contents of the eight selected β-tubulin isotypes were also analyzed in 79 cancer samples corresponding to seven different tumor types (kidney, lung, colon, prostate, ovary, breast, and larynx; see Table I for details). The percentages that each isotype represented in the different tumors are shown in Fig. 2c. *TUBB* was the most abundant isotype in most tumors, followed by *TUBB2C, TUBB2A, TUBB6*, and *TUBB3* (with 47, 38, 8.9, 3.1, and 2.2%, respectively) and with *TUBB4, TUBB2B*, and *TUBB1* levels below 0.5% of the total β-tubulin. Comparing with the nontumoral tissue, the isotype that had the greatest tumoral expression increase was *TUBB3*, which showed a 43- and 71-fold change in breast and lung cancers, respectively (compare *TUBB3* portions in Fig. 2c 2nd and 5th bars with Fig. 2a 11 th and 14th bars), even though *TUBB3* represented in average less than 5% of total tumoral β-tubulin content. *TUBB* also showed a considerable overexpression in kidney and lung tumors (3.8- and 5.9-fold increase). In contrast, *TUBB6* expression was largely decreased in all tumors except for kidney, and *TUBB2C* expression decreased in most tumors.

Since several samples of each tumor type were available, we also studied the variation in the expression among the different tumoral samples. In Fig. 3, the percentages that each isotype represented in the different types of tumors is shown. *TUBB* (Fig. 3a) was the most abundant isotype followed by *TUBB2C* (Fig. 3c), in general mimicking the

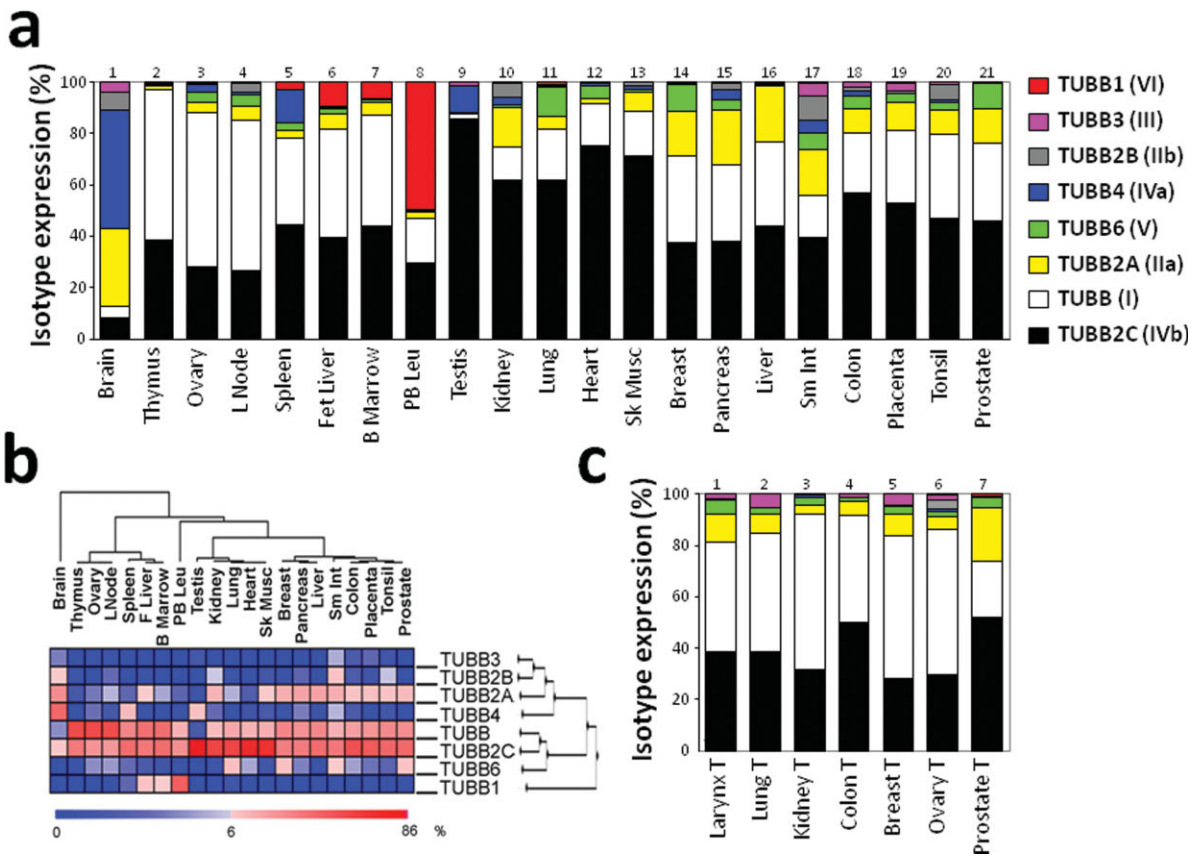

Fig. 2. Relative β-tubulin isotypes mRNA expression in nontumoral and tumoral tissues. (**a**) The mRNA content of the eight isotypes was measured for each nontumoral tissue and the fraction that each isotype represented was calculated and represented in bar graphs. The mRNA quantity of each isotype was calculated as described in Materials and Methods section. (**b**) Unsupervised clustering of the tissue specific relative expression data for the β-tubulin isotypes in nontumoral tissues was performed using the hierarchical Furthest Neighbor (complete linkage) analysis from the GEPAS 4.0 software (http://gepas.bioinfo.cipf.es/) using as distance for gene clustering the correlation coefficient function (offset of 0) c. The percentage of each β-tubulin isotype in different tumors is shown as the mean isotype mRNA expression of each tumor type. The number of samples for each tumor type were 6 kidney, 5 lung, 7 colon, 10 prostate, 4 ovary, 27 breast, and 20 larynx tumoral samples.

normal tissue distribution. *TUBB* maximum and minimum levels occurred in an ovary and a prostate tumor, respectively, with 80 and 12% of the total β-tubulin, while for *TUBB2C* maximum levels (78%) corresponded to a breast sample and the minimum levels (7%) to an ovary tumor. *TUBB3* (Fig. 3d) was the isotype with the largest variation and with levels that reached up to 18, 16, 7.5, and 4.9% of the total β-tubulin in breast, lung, ovary, and larynx tumor samples, respectively. *TUBB2A* (Fig. 3b) and *TUBB6* (Fig. 3e) maximum levels corresponded to breast samples (49 and 14%, respectively).

We then compared the expression of the most relevant tumoral isotypes in the matched nontumoral/ tumoral samples (10 prostate and 4 breast cases, see Table I), we found that *TUBB* and *TUBB6* expression was significantly decreased in prostate tumors ($P = 0.06$ and 0.005, respectively; Supp. Info. Figs. 1a and 1e). In breast tumors only, *TUBB2A* showed a significantly different expression when compared to the matched normal samples (4-fold decreased tumoral expression, $P = 0.0002$; Supp. Info. Fig. 1b). Three of the four matched breast tumoral samples showed increased *TUBB3* expression, however, probably due to the small number of samples analyzed, the results were not significant, despite an average of 1000-fold *TUBB3* tumoral increase.

We then determined whether the expression of the different isotypes could be associated to a specific tumor subtype or differentiation degree in breast and larynx tumors (for which we had the largest number of cases: 27 and 20, respectively). Regarding breast cancer, *TUBB2C* showed significantly higher expression in the more aggressive ER negative tumors than in ER positive ones ($P = 0.008$; Fig. 4). *TUBB* showed a similar tendency, although the differences did not reach significance and *TUBB3* had in average a higher expression in ER negative tumors, and the three tumors with the highest expression corresponded to this type, although the differences were not significant (Supp. Info. Fig. 2). Regarding larynx tumors, none of the isotypes analyzed showed a significantly different expression between the 15 well differentiated and the 5 moderately to poorly differentiated tumors or between the 3 tumors that received cisplatin before the surgery and the 17 tumors that did not (data not shown).

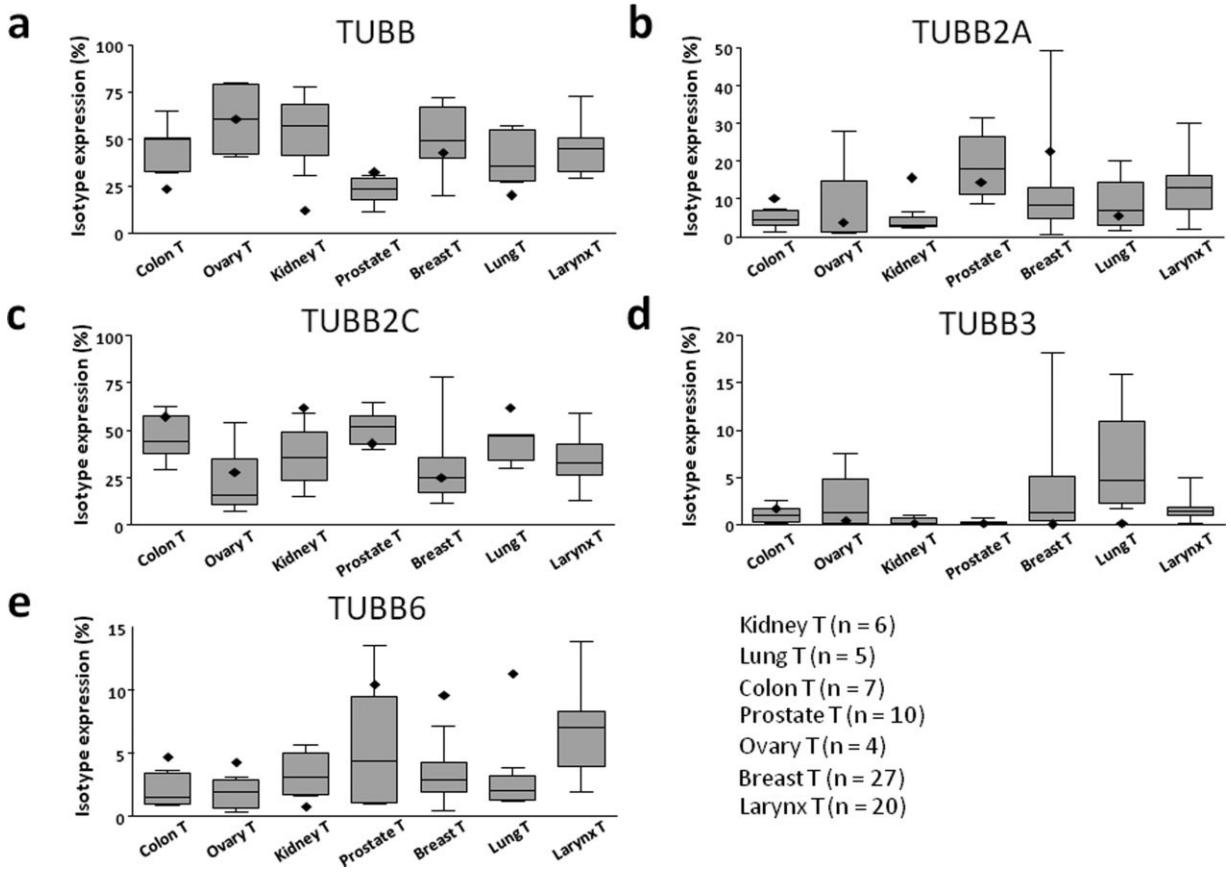

Fig. 3. Tumoral variation in β-tubulin isotypes mRNA expression. The variation in the percentage of each β-tubulin isotype (calculated using the eight different isotypes listed in Table II) among the different tumors is shown in a box plot. The boxes showing the interquartile range, the horizontal line representing the median value for each group, and the whiskers extending to the minimum and maximum values. The diamonds show the expression of the corresponding nontumoral tissue. The number of samples of tumor type are shown at the bottom right.

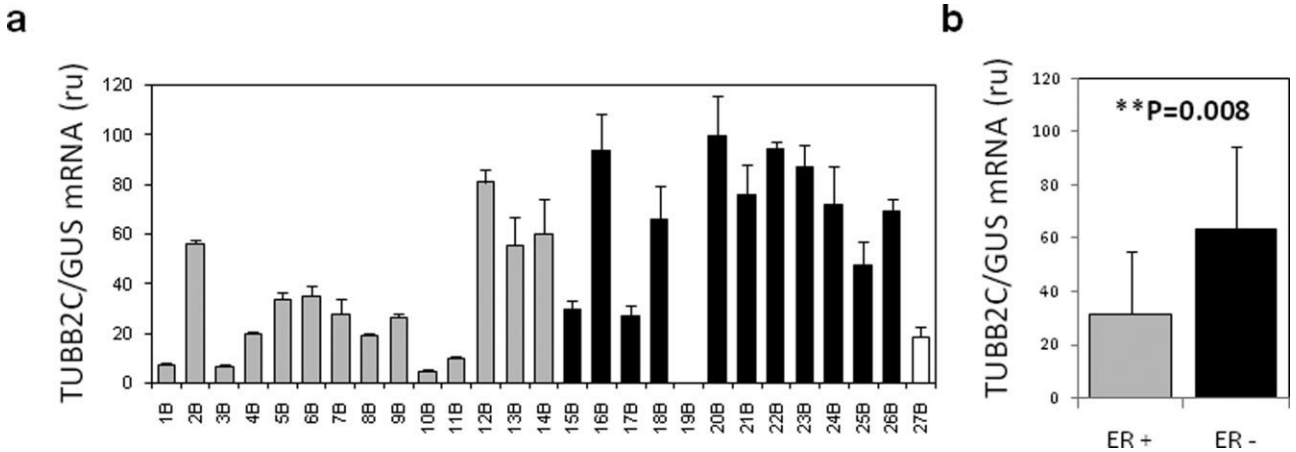

Fig. 4. Expression of *TUBB2C* mRNA in human breast tumors with different estrogen receptor (ER) status. (**a**) The mRNA amounts of *TUBB2C* in 27 breast tumors are shown. (**b**) *TUBB2C* mRNA average values. The mRNA expression of ER positive tumors was statistically significant different from that of ER negative tumors ($P = 0.008$). The gray bars correspond to ER positive tumors, the black bars to ER negative tumoral samples and the white bar to a tumoral sample with unknown ER status.

Discussion

β-tubulins play a crucial role in mitosis and are the targets of several widely used anticancer drugs, however, there is scarce information concerning the β-tubulin isotypes expression in nontumoral and tumoral tissues. Whether this could have an impact on the microtubule binding agents' efficacy and toxicity it is still unknown. In this study, we have

developed a real-time quantitative RT-PCR technique able to accurately determine the mRNA expression of the eight most abundant human β-tubulin isotypes. This technique is able to detect the eight isotypes without cross reactions (i.e. class IVa/IVb and also class IIa/IIb proteins share the same C-terminals, resulting in antibodies that cannot differentiate these isotypes). This technique was used to establish the β-tubulin mRNA expression in a large number of nontumoral and tumoral tissues.

Quantitatively inferring β-tubulin protein levels from mRNA should be done with caution. Nevertheless, general conclusions can be drawn based on mRNA data (e.g. lack of mRNA results in lack of protein, and mRNA increases/decreases in tumoral tissues will likely result in alterations at protein level). This is further supported by good correspondence between β-tubulin mRNA and protein levels [Shalli et al., 2005; Hiser et al., 2006]. Furthermore, in bovine brain, the relative protein expression of the different β-tubulin isotypes was 3, 58, 25, 13, 0, and 0% for class I, II, III, IV, V, and VI, respectively [Cleveland et al., 1990], while the human brain mRNA content found in this work for the corresponding genes was 4, 37, 4, 55, 0, and 0%. Concerning cancer cells, a recent work measuring protein levels by quantitative methods in human cell lines from colon, breast, lung, and ovarian tumors found 69, 30, and 2% for class I, IVa/IVb, and III, respectively [Hiser et al., 2006]. This is in agreement with previous reports using different protein quantification techniques [Verdier-Pinard et al., 2005] and also with the mRNA data from this work in colon, breast, lung, and ovarian tumors with an average *TUBB*, *TUBB2C/4*, and *TUBB3* mRNA levels of 51, 37, and 3%, respectively. These data show that *TUBB* is the major isotype in the tumor types tested, in agreement with earlier reports on the relative expression of β-tubulin isotypes in cancer cell lines, xenograft models, and ovarian tumors [Giannakakou et al., 1997; Nicoletti et al., 2001; Ohishi et al., 2007]. Previous studies using lung tissues and breast tumors [Dozier et al., 2003; Cucchiarelli et al., 2008], corroborate the low expression of isotypes IVa and VI, however, the highest mRNA expression in these studies did not correspond to isotype I, but to class V and II for NSCLC and to both class I and V for breast cancer. The discrepancy between the exact percentages identified for each isotype between the study by Nicoletti et al. and our work is likely due to the semi-quantitative nature of the expression data in the earlier report. On the other hand, the discrepancy for class V and II expression with the studies by Cucchiarelli et al. [2008] and Dozier et al. [2003] might be due to differences in the qRT-PCR design, which is shared by both studies (e.g. using gene-specific primers for each isotype in the RT, possible cross-reactions between isotypes or differences in the length of the amplified PCR products).

Concerning the tissue specificity, among the ubiquitous β-tubulin isotypes, *TUBB* and *TUBB2C* were the most abundant isotypes in 19 of the 21 tissues here studied (brain and leukocytes being the exception). In average, together they represented more than 75% of the total β-tubulin content. The contribution of the also ubiquitous isotype *TUBB6* was much smaller, representing in average only 3% of the total β-tubulin content. The amounts of *TUBB2C* exceeded those of *TUBB* in several tissues, its highest expression occurring in testis, as already suggested by studies with homologue proteins [Havercroft and Cleveland, 1984]. *TUBB1* expression was restricted to hematopoietic tissues, while *TUBB2A, TUBB2B, TUBB3*, and *TUBB4* were expressed at very high levels in brain. The large number of brain isotypes likely reflecting the importance of the extensive neuronal cytoskeleton for the diverse functions of the human neurons. When the expression of the so called "neuronal-specific" isotypes was studied in other tissues, it became clear that some of these isotypes were also present at relevant amounts in specific tissues. *TUBB2A* was the isotype with the highest expression outside the brain, followed by *TUBB4, TUBB2B*, and *TUBB3* (see Fig. 2a). Similarly, Arai et al. [2002] showed that brain class II was expressed at low levels in a broad range of tissues.

Interestingly, we noticed that human *TUBB2A* and *TUBB2B* genes are located in tandem in chromosome 6p and encode proteins that only differ in two amino acids. When we examined *Pan troglodytes* genome, *TUBB2A* was not found, suggesting that this gene was recently acquired in humans by duplication of *TUBB2B.* From the two amino acids differentiating the two human class II proteins, at positions 55 Thr/Ala and 201 Cys/Ser, the latter is conserved in all istoypes except for *TUBB2A*, supporting that this is a recent gene. The functional implications of the duplication and the amino acid changes in class IIa protein are unknown, but the similarity between IIa and IIb proteins and the neuronal-specificity suggests a redundancy. On the other hand, the expression of human *TUBB2A* surpassed that of *TUBB2B* in most normal tissues examined, and this difference greatly increased in tumors.

Concerning the β-tubulin expression in tumors, we found that *TUBB* (class I) was the major isotype, followed by *TUBB2C* (class IVb). The antitubulin agents taxol and taxotere exert their antitumoral effect through β-tubulin binding and are clinically used as a first choice treatment for breast, ovary, and lung cancers; however, taxanes are not clinically effective for colon and kidney cancers. Interestingly, *TUBB* was the major isotype in ovary, breast, and lung tumors, but it was also highly expressed in colon and kidney cancers. This suggests that *TUBB* tumoral expression is not the only requirement for taxanes effectiveness, and that other factors such as β-tubulin post-translational modifications or binding of MAPs could also be critical for the drugs' sensitivity [Verhey and Gaertig, 2007; Luchko et al., 2008]. Concerning *TUBB2C*, both mRNA and protein contents were increased in breast cancer cell lines resistant to docetaxel [Shalli et al., 2005] and knockdown of this isotype in vitro increased the sensitivity to *Vinca* alkaloids [Gan and Kavallaris, 2008]. However, overexpression or knockdown of β-tubulin IVb did not seem to influence paclitaxel response in different in vitro models [Blade et al., 1999; Gan and Kavallaris, 2008]. In this work, we found an increased expression of *TUBB2C* in ER

negative compared to ER positive breast tumors (Fig. 4), which could result in differences in drug response. Indeed, several clinical trials have suggested that breast cancer patients with different ER status show differential responses to taxane combined chemotherapy [Berry et al., 2006; Conforti et al., 2007]; however, there are also contradictory results [Andre et al., 2008], which indicate that further research is necessary. The expression of the neuronal-class III has been associated in vitro to altered sensitivity to antitubulin agents [Hari et al., 2003; Kamath et al., 2005] and there is also mounting clinical evidence that it could be involved in tumoral resistance to taxanes [Hasegawa et al., 2003; Mozzetti et al., 2005; Ferrandina et al.,2006, 2007; Seve et al., 2005,2007a,2007b]. In this work, *TUBB3* contribution to the total β-tubulin content in breast, ovary and lung tumors was in average small, but much greater than that in colon and kidney tumors (Figs. 2c and 3d). *TUBB3* reached up to 18 and 16, 7.5% of the total β-tubulin in breast, lung, and ovary cancer samples, respectively, making feasible that, in these cases, *TUBB3* could play role on taxanes sensitivity. Another characteristic of the tumors here studied, was a decreased expression of *TUBB6*, except for kidney (see Figs. 2c, 3e and Supp. Info. Fig. 1e). Class V has been shown in vitro to confer unique properties to the microtubules, disrupting the organization, increasing the fragmentation and resulting in disruption of mitotic spindle assembly and block proliferation [Bhattacharya and Cabral, 2004]. However, normal tissues express various amounts of *TUBB6* (Fig. 2a) and whether *TUBB6* plays an in vivo function or if there is a biological meaning for tumoral *TUBB6* alteration, it remains unknown.

The complex expression patterns of the multiple β-tubulin genes and several in vitro experiments [Banerjee et al., 1990; Panda et al., 1994; Derry et al., 1997] suggest a different functionality for the isotypes; however, there is still no clear in vivo proof supporting this. Alternatively, gene-duplications might have formed multiple genes encoding functionally equivalent proteins that have evolved to possess different regulatory sequences that are activated differentially. Thus, further studies are required to fully understand the biological role of the β-tubulin proteins. This knowledge is crucial to undertake rational studies aimed at the identification of factors influencing tumoral resistance to antitubulin drugs. On the other hand, the differences between the proteins could be directly exploited in the design of antitubulin agents binding to specific β-tubulin isotypes and with lower toxicity. For example, drugs with low affinity for the neuronal specific class IVa (*TUBB4),* which is not expressed in tumors, might retain their tumoral efficacy while showing a lower neurotoxicity, which is a common and serious side effect of the microtubule binding drugs. Furthermore, the recent work by Escuin et al. [2009] has proven that this is feasible clinically, by demonstrating that the low affinity of 2-methoxyestradiol (a microtubule-depolymerizing agent) for the hematopoietic-specific class VI β-tubulin (see Figs. 1b and 2a, 7th and 8th bars) is correlated with the lack of hematologic toxicity in patients receiving 2ME2-based chemotherapy. All together, this work provides key data concerning the normal and tumoral distribution of the most relevant β-tubulin isotypes in different human tissues. These expression patterns could help in the design of novel isotype-specific drugs that could minimize tumoral resistance, while decreasing the toxicities in the patients.

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The miR-200 family controls β-tubulin III expression and is associated with paclitaxel-based treatment response and progression-free survival in ovarian cancer patients

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Abstract

Ovarian cancer remains one of the leading causes of cancer deaths. Thus, new biomarkers predictive of response to the standard paclitaxel–carboplatin treatment are needed to improve chemotherapy strategies. MicroRNAs have the potential to modify drug outcomes. Based on this, we have demonstrated in this study that patients with a high expression of the miR-200 family show low levels of β-tubulin class III in ovarian carcinoma. In addition, we have established the clinical relevance of these microRNAs for ovarian cancer patients' treatment response and survival. In a well-characterized series of 72 ovarian carcinomas, the expressions of miR-141, miR-200a, miR-200b, miR-200c, and miR-429 were quantified by quantitative reverse transcription-PCR, and the protein content of β-tubulin isotypes I, II, and III was determined by immunohistochemistry. The relationship between these microRNAs, β-tubulin expression, response to paclitaxel-based treatment, progression-free survival (PFS) and overall survival was determined. While isotype I had constant high levels, protein expression of β-tubulins II and III was mutually exclusive. Low tumoral miR-200 expression was significantly associated with high β-tubulin III protein content (P values range, 0.047–$<$0.0001), and patients without complete response (CR) had lower miR-200c levels than patients with CR (hazard ratio (HR)$=$1.43, 95% confidence interval (CI)$=$1.02–1.99, $P=0.037$, multivariate analysis). Additionally, low miR-200 family expression had a trend toward poor PFS (HR$>$2.0, P values 0.051, 0.054, and 0.079 for miR-200c, miR-141, and miR-429 respectively, multivariate analysis). In conclusion, miR-200 family members affect the final β-tubulin III protein content of ovarian carcinomas. Furthermore, these microRNAs might constitute the biomarkers of response to paclitaxel-based treatments and relapse/progression of advanced stage ovarian carcinoma patients.

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Introduction

Ovarian cancer is the leading cause of death for gynecologic malignancies (Jemal *et al*. 2009). More than 70% of patients are diagnosed at late stages of the disease and, although the current standard treatment combining surgery with chemotherapy (mainly based on paclitaxel–carboplatin regimens) is efficient in almost 80% of cases, the 5-year survival rate is low due to the high incidence of recurrence and to the ultimate resistance to taxanes and/or platinum-based drugs (Ozols *et al*. 2003, Heintz *et al*. 2006). New biomarkers predictive of treatment response urgently need to be identified in order to improve chemotherapy strategies.

First-line treatment for advanced ovarian cancer consists of a combination of paclitaxel and carboplatin (Ozols *et al*. 2003, Omura 2008). Carboplatin is equally efficient but less toxic and easier to administer than cisplatin, thus, it replaced cisplatin as the standard treatment for ovarian cancer (Ozols *et al*. 2003, Omura 2008). Paclitaxel is an antimitotic drug, which alters the dynamics of the cellular microtubules, which maintain cellular structure and are essential for diverse cellular functions such as cell cycle, cell signaling, and intracellular trafficking (Seve & Dumontet 2008). Paclitaxel binds to β-tubulin, leading to cellular microtubules stabilization, mitotic arrest, and finally to cell death. In humans, there are at least eight different isotypes of β-tubulin, which exhibit an altered expression pattern in tumoral tissue (Leandro-Garcia *et al*. 2010). In ovarian carcinoma, high protein levels of classes I and IV, intermediate levels of class III, and low levels of class II β-tubulin have been reported (Ohishi *et al*. 2007). High tumoral β-tubulin III expression has been associated with worse survival in non-small cell lung cancer (Rosell *et al*. 2003, Seve *et al*. 2005), breast (Seve & Dumontet 2008), head and neck (Koh *et al*. 2009), and ovarian cancer (Ferrandina *et al*. 2006), although Aoki *et al*. (2009) reported a better survival for patients with ovarian clear cell adenocarcinoma positive for class III expression. In ovarian cancer, β-tubulin III has also been associated with worse treatment response (Kavallaris *et al*. 1997, Mozzetti *et al*. 2005, Umezu *et al*. 2008). On the other hand, absence of class II β-tubulin expression has been associated with advanced stage and short progression-free survival (PFS) in ovarian tumors (Ohishi *et al*. 2007).

The molecular mechanisms leading to the upregulation of class III β-tubulin in tumors remain largely unknown. Hypoxia-inducible factor 1 seems to play a role in ovarian carcinomas (Raspaglio *et al*. 2008), and epigenetic modifications have been suggested to be a contributing factor in ovarian tumors (Izutsu *et al*. 2008) and melanoma cells (Akasaka *et al*. 2009). Recent studies suggest an important role of microRNAs, specifically, Cochrane *et al*. (2009, 2010) demonstrated that miR-200c had a binding site in the 3′ untranslated region (UTR) of class III β-tubulin and that overexpression of miR-200c in cell lines decreased β-tubulin III protein content and restored sensitivity to microtubule-targeting agents. Interestingly, the miR-200 family, formed by five microRNAs (miR-141, miR-200a, miR-200b, miR-200c, and miR-429) and located in two clusters in the genome, is involved in the epithelial to mesenchymal transition (EMT) through regulation of E-cadherin expression via suppression of ZEB1 and ZEB2 (Gregory *et al*. 2008, Korpal *et al*. 2008, Park *et al*. 2008). In ovarian cancer, a recent study with 55 advanced tumor samples showed that a high expression of miR-200a, miR-200b, and miR-429 was associated with improved survival (Hu *et al*. 2009). Nevertheless, another study including 20 serous carcinomas found that high expression of the miR-200 family members significantly correlated with poor prognosis (Nam *et al*. 2008). Thus, although there is evidence suggesting that the miR-200 family might play a key role in the response to microtubule-binding drugs and ovarian cancer survival, the relevance of these microRNAs as clinical markers for patients is largely unknown.

In this study, we quantified the expression of the miR-200 family in a well-characterized series of 72 epithelial ovarian tumors and examined their contribution to the protein expression of β-tubulin isotypes I, II, and III. We also investigated the impact of these microRNAs on the patients' response to and survival following paclitaxel-based therapy. The data provided in this study improved our comprehension of treatment failures in ovarian carcinoma and indicated the miR-200 family as a potential novel target for improved treatment strategies.

Materials and methods

Patient selection

This study included 72 formalin-fixed and paraffin-embedded ovarian carcinoma samples from patients treated at the Hospital Universitario La Paz (HULP), Madrid, Spain. All patients underwent a baseline computed tomography (CT) scan and exploratory laparotomy for diagnosis, staging, and debulking when feasible. All patients received a platinum/taxane-based chemotherapy for at least six cycles. Patients were divided into stages according to the International

Federation of Gynecology and Obstetrics (FIGO) classification. Optimal debulking was defined as ≤ 1 cm (diameter) residual disease. A complete response (CR) was defined as absence of all clinical/radiographic evidence of disease. In addition, a second-look laparotomy (SLL) was performed on most of the patients who achieved a CR after planned treatment and all of them who were optimally debulked. In patients who achieved a CR after the planned treatment and did not accept an SLL or for whom this procedure was not feasible, and in patients with a partial response (PR), a second CT scan was performed 1 month after the first evaluation to confirm the response. Follow-up data were obtained by retrospective chart review. PFS was defined as the time interval between the start of the treatment and the first confirmed sign of disease recurrence or progression. Overall survival (OS) was defined as the time interval between the start of the treatment and the date of death or end of follow-up. Approval for the study was obtained from the local ethics committee. Relevant clinicopathological data of the patients are shown in Table 1.

Treatment response was studied in a homogeneous subgroup of 57 patients with both advanced tumor stage (FIGO stages III and IV) and serous carcinoma histology. In this subgroup of patients, 84 and 16% of the tumors corresponded to III and IV stages respectively. Regarding tumor grade in this subset of tumors, 61, 32, and 7% of the tumors were grades 3, 2, and 1 respectively. The clinical response to the treatment, debulking status after surgery, and survival data in this subgroup of patients are presented in Table 2.

Table 1 Clinicopathological data of the ovarian cancer patients[a]

	All cases ($n=72$)		Serous III/IV ($n=57$)[b]	
	n	**%**	***n***	**%**
Age (years)				
Median	57.0		54.0	
(minimum–maximum)	(35–85)		(35–85)	
Histological subtype				
Serous carcinoma	57	80	57	100
Clear cell carcinoma	6	8	–	–
Endometrioid carcinoma	4	6	–	–
Mucinous carcinoma	3	4	–	–
Mixed endometrioid–clear cell carcinoma	1	1	–	–
Mixed endometrioid–serous carcinoma	1	1	–	–
FIGO stage				
I	3	4	–	–
II	5	7	–	–
III	54	75	48	84
IV	10	14	9	16
Tumor grade				
Well differentiated (grade 1)	8	11	4	7
Moderately differentiated (grade 2)	24	33	18	32
Poorly differentiated (grade 3)	39	54	35	61
Unknown	1	1	–	–

FIGO, International Federation of Gynecology and Obstetrics.
[a]All patients included in the study were Caucasian females.
[b]Clinicopathologial characteristics of the subset of 57 patients with FIGO stages III and IV and serous ovarian carcinoma.

Tissue microarray construction

Representative areas of the tumors were selected on hematoxylin and eosin-stained sections and marked on individual paraffin blocks. Two tissue cores (1 mm in diameter) were obtained from each specimen. The tissue cores were arrayed into a receptor paraffin block using a tissue microarray workstation (Beecher Instruments, Silver Spring, MD, USA) as previously described (Hardisson *et al*. 2003). A hematoxylin and eosin-stained section of the array was reviewed to confirm the presence of morphologically representative areas of the original lesions.

Immunohistochemistry

Immunohistochemistry was performed on 4 μm sections of formalin-fixed, paraffin-embedded tissues. Briefly, the tissue sections were deparaffinized and rehydrated in water, after which antigen retrieval was carried out by incubation in EDTA solution, pH 8.2 at 50 °C for 45 min in an autoclave. Endogenous peroxidase and nonspecific antibody reactivity were blocked with peroxidase-blocking reagent (Dako, Glostrup, Denmark) at room temperature for 15 min. The sections were then incubated for 60–90 min at 4 °C with the following antibodies: class I β-tubulin (clone SAP.4G5, Sigma–Aldrich, dilution 1:100), class II β-tubulin that recognizes classes IIa and IIb β-tubulins, which differ in one single amino acid, (clone 7B9, Covance, Emeryville, CA, USA, dilution 1:100), and class III β-tubulin (clone TUJ-1, Santa Cruz Biotechnology, Heidelberg, Germany, dilution 1:200). Detection was performed with Envision Plus Detection System (Dako).

Immunohistochemical results were evaluated and scored by one pathologist (D H) blinded to the clinical data of the patients. Immunoreactivity was scored by estimating the percentage of tumor cells with cytoplasmic immunostaining, regardless of intensity. The intensity of the immunostaining was not considered

Table 2 Clinical characteristics of patients with International Federation of Gynecology and Obstetrics (FIGO) stages III and IV and serous ovarian carcinoma

	n	%
Response to treatment		
Complete response	38	67
Partial response	11	19
Stable disease	3	5
Progressive disease	4	7
Unknown	1	2
Pathological response		
Complete response	14	25
Stable disease	10	17
Unknown	33	58
Debulking status		
Optimal (<1 cm)	24	42
Suboptimal (>1 cm)	12	21
Unknown	21	37
Relapse/progression		
Yes	47	83
No	8	14
Unknown	2	3
Progression-free survival (months)		
Median	16.0	
(minimum–maximum)	(1–128)	
Deceased		
Yes	35	61
No	19	33
Unknown	3	5
Overall survival (months)		
Median	35.1	
(minimum–maximum)	(1–128)	

since in our study the intensity of the immunostaining in the positive tumors was predominantly high, precluding discrimination between the samples. Class I β-tubulin expression was not categorized since the expression levels of all samples were consistently high. In the tissue sections, negative controls were used with only the secondary, replacing the primary antibody.

RNA isolation and real-time quantitative reverse transcription-PCR

Tissue sections previously stained with hematoxylin and eosin were reviewed by an experienced pathologist. Eligible samples included at least 80% of tumor cells and no large necrotic areas. Four to eight 4 μm sections were used for RNA isolation with the Masterpure RNA Purification kit (EPICENTRE Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions. For microRNA quantitative reverse transcription (qRT)-PCR, 25 ng total RNA were reverse transcribed using the miRCURY LNA First-Strand cDNA kit (Exiqon A/S, Vedbaek, Denmark) and the miRCURY LNA microRNA Primer Sets (Exiqon) corresponding to hsa-miR-141, hsa-miR-200a, hsa-miR-200b, hsa-miR-200c, hsa-miR-429, and the control primer set 5S rRNA, according to the manufacturer's instructions. Negative controls with reaction mix without reverse transcriptase were included for the different microRNAs studied. Real-time qPCR was performed with the Sequence Detection System 7900HT (Applied Biosystems, Carlsbad, CA, USA) using the miRCURY LNA SYBR Green Master Mix (Exiqon), following the manufacturer's instructions. The amplification conditions consisted of an initial step at 95 °C for 10 min, followed by 50 cycles of 20 s at 95 °C and 1 min at 60 °C. Negative controls were included in all PCRs, and all assays were performed in triplicate. The $\Delta\Delta C_t$ method was used for the calculation of the different amounts of mRNA (Livak & Schmittgen 2001). Normalization was carried out with the endogenous control 5S ribosomic RNA. The relative abundance of different miR-200 family members cannot be precisely estimated because the hybridization characteristics of the different qRT-PCR probes could vary, however, approximate conclusions can be drawn due to the large differences found (Supplementary Figure 3, see section on supplementary data given at the end of this article).

Statistical analysis

All statistical analyses were carried out using SPSS version 17.0 statistical software (SPSS Inc., Chicago, IL, USA). The association between the expression levels of the five members of the miR-200 family and continuous demographic variables (such as age) was determined by the Pearson coefficient. β-Tubulins II and III protein expression, treatment response, pathological response, mortality, relapse, and tumoral characteristics (histology subtype, differentiation grade, and tumor stage) were analyzed as categorical variables. Similar to previous publications, protein expression of β-tubulins II and III was used as a binary variable (low expression versus high expression) using 75% of positive cells as the cutoff (Ohishi *et al*. 2007). The response to treatment was divided into two categories: patients with CR and those with PR, stable disease, and disease progression grouped together. Associations between the response to treatment and clinical variables (histology subtype, differentiation grade, tumor stage, and patient's age) were evaluated with χ^2 test, Fisher's exact test, and Student's *t*-test, when appropriate. P values <0.05 were considered statistically significant and all of them are two-sided. To analyze the associations between miR-200 expression and β-tubulin content and treatment

response, since the tumoral miR-200 family content followed a normal distribution (Kolmogorov–Smirnov test), Student's *t*-tests were used, applying the Welch correction when the S.D.s differed significantly between the groups. To further analyze miR-200 expression and response to treatment, logistic regression was applied and the hazard ratio (HR) was estimated, adjusting for relevant clinicopathological variables. To analyze miR-200 expression and PFS and OS, the univariate analysis was carried out by using the Kaplan–Meier plots coupled to log-rank test and univariate Cox regression model was applied for the HR estimation. The multivariate Cox proportional hazards regression model was used to evaluate the prognostic significance of the microRNA adjusted by clinicopathological variables.

Results

Predicted binding sites of miR-200b/200c/429 in the β-tubulin 3′ UTR and immunohistochemical determination of β-tubulin isotypes I, IIa, and III expression

Recently, *in vitro* studies have shown that the 3′ UTR region of class III β-tubulin has a miR-200c-binding site. The important role that this microRNA plays in metastasis and the high degree of conservation among β-tubulin isotype functions and genetic structure led us to further explore in tumor samples the correlation between the expression of the tubulin isoforms and the miR-200 family. By means of an *in silico* analysis (http://www.targetscan.org/), miR-200b/200c/429-binding sites were predicted in the 3′ UTR not only of class III β-tubulin but also of classes I and IIa, while no binding sites were predicted in the rest of the human β-tubulin isotypes. The miR-200b/200c/429 microRNA-binding site was broadly conserved among vertebrates for classes I and III β-tubulins, but poorly conserved for class IIa. The *in silico* analysis did not predict miR-141 and miR-200a binding, due to one nucleotide difference in the seed sequence (Supplementary Figure 1, see section on supplementary data given at the end of this article), but there is evidence for miR-200 family common targets.

We then determined the protein expression of classes I, II, and III β-tubulins in 72 ovarian cancer samples by immunohistochemical analysis (Fig. 1). The protein staining showed substantial differences for the different isotypes, while class I protein expression showed no variation among the cases, β-tubulins II and III exhibited substantial intersample differences. All samples exhibited a very strong staining for class I β-tubulin; class II protein was absent in 46 (69%) tumors, while 15 cases had low and 6 cases had a high protein expression (22 and 9% of the tumors respectively). Class III protein was absent in 34 (48%) cases, while 29 (41%) had low and 8 (11%) had a high protein expression (Supplementary Figure 2A, see section on supplementary data given at the end of this article). With regards to the correlation between the different isotypes, classes II and III β-tubulin expression proved to be mutually exclusive events, with samples exhibiting a high β-tubulin class III content lacking isotype II expression and vice versa (Supplementary Figure 2B, see section on supplementary data given at the end of this article).

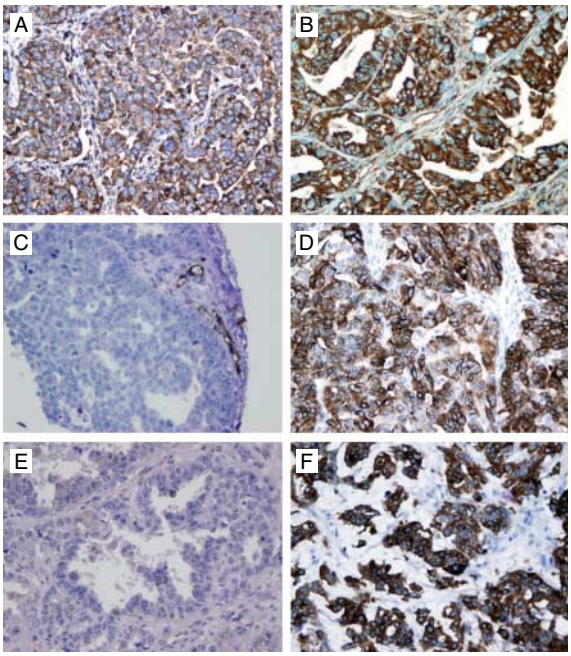

Figure 1 Protein expression of β-tubulin isotypes I, II, and III in ovarian carcinomas. Isotype I exhibited a high expression in all cases analyzed with minimal differences among samples (A) and (B). Illustrative cases with low (C) and high (D) β-tubulin isotype II expression. Illustrative cases with low (E) and high (F) β-tubulin isotype III expression. All cases shown correspond to serous carcinomas.

Expression of the miR-200 family is associated with tumoral β-tubulin III protein expression

To investigate whether the miR-200 family could regulate β-tubulin isotypes I, II, and III, we measured the expression of these microRNAs in ovarian cancer samples. The expression levels found were variable, with miR-200c expressed at the highest level, then, miR-200b, miR-200a, and finally miR-429 and miR-141, which were expressed at similarly low-expression levels (Supplementary Figure 3, see section

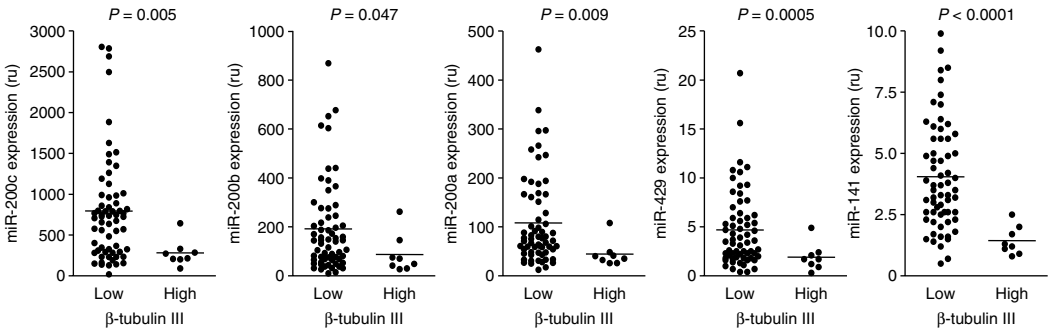

Figure 2 Tumors with high levels of β-tubulin III protein have significantly decreased miR-200 expression. Samples with high isotype III expression (more than 75% positive cells) showed a significantly lower miR-200c, $P=0.005$; miR-200b, $P=0.047$; miR-200a, $P=0.009$; miR-429, $P=0.0005$; and miR-141, $P<0.0001$ expression compared with samples with low isotype III expression. MicroRNAs are shown in the figure according to the expression levels. To express the microRNAs content as whole numbers, their expression was multiplied by 100 and expressed as relative units (ru).

on supplementary data given at the end of this article). As expected, correlations were found among the miR-200 family members, with miR-141/miR-200a and miR-200a/miR-200b showing the highest correlation and miR-429 the lowest (Supplementary Table 1, see section on supplementary data given at the end of this article).

We then determined whether the miR-200 family could regulate the protein expression of β-tubulin isotypes I, II, and III. We found a statistically significant association between class III β-tubulin protein expression and the tumoral content of all miR-200 members (Fig. 2). According to this, the ovarian tumors with low miR-200 expression exhibited high levels of class III protein, suggesting that the absence of these microRNAs in the tumor results in lack of class III β-tubulin degradation and accumulation of high levels of the protein. The strongest associations corresponded to miR-141, miR-429, and miR-200c ($P<0.006$) among which miR-200c showed the highest expression. No association was found between protein levels of β-tubulins I and II with miR-200 family expression (data not shown).

miR-200c expression determines the response to paclitaxel-based chemotherapy in serous ovarian carcinoma patients

Owing to the importance of tumor type and stage, we selected a homogenous subgroup of patients with serous carcinomas and advanced tumor stage (FIGO stages III and IV) to study whether the miR-200 family could influence clinical response to treatment: fifty-seven patients met these inclusion criteria. In these samples, we did not find statistically significant associations between the expression of classes I, II, or III β-tubulin content and response to treatment, relapse, or survival of the patients. Notwithstanding this, we found a statistically significant association between miR-200c expression and response to treatment ($P=0.0027$ with t-test; HR = 1.43, 95% CI = 1.02–1.99, $P=0.037$ with logistic regression multivariate analysis, Table 3). The patients who did not achieve a complete clinical response had lower miR-200c levels than those patients with CRs. A significant association was also found between miR-200c and pathological response using the t-test ($P=0.045$), although it did not reach significance in a logistic regression multivariate analysis (HR = 1.45, 95% CI = 0.94–2.25, $P=0.094$). With respect to the number of recurrence and mortality events, low expression of miR-200c was associated with recurrence (odds ratio (OR) = 1.17, 95% CI = 1.01–1.34, $P=0.030$), while no significant association was observed for mortality (OR = 1.11, 95% CI = 0.97–1.28, $P=0.128$; Table 3). miR-200c expression did not show any association with other clinicopathological characteristics. No association with treatment response was found for any of the other miR-200 family members.

miR-200 family expression is associated with prognosis in serous ovarian carcinoma patients

Of the miR-200 family, only miR-429 expression showed a statistically significant association with the recurrence-free survival and OS of the patients (HR = 2.01, 95% CI = 1.11–3.66, $P=0.021$ and HR = 2.08, 95% CI = 1.03–4.20, $P=0.041$; Fig. 3). The PFS rate at 12 months post treatment was 85% in the group of high miR-429 expression and 48% for those with low expression. For miR-200c, the PFS rate at 12 months was 73 and 54% for high and low expressors respectively. After the multivariable analysis, adjusting for relevant clinicopathological

Table 3 miR-200c expression and response to paclitaxel–carboplatin chemotherapy

	Number of samples	miR-200c		Difference of means	Logistic regression	
		Mean	95% CI (ru)	*P* value[a]	HR (95% CI)	*P* value[b]
Response						
CR	37	9.1	(6.8–11.3)	**0.0027**[c]	**1.43 (1.02–1.99)**	**0.037**
No CR (PR, SD, PD)	17	4.8	(3.1–6.5)			
Pathological response						
CR	14	8.0	(5.6–10.3)	**0.045**	1.45 (0.94–2.25)	0.094
No CR	10	4.6	(2.0–7.1)			
Recurrence						
No	8	11.8	(3.1–20.6)	0.243[c]	**1.17 (1.01–1.34)**	**0.030**
Yes	45	7.0	(5.5–8.5)			
Deceased						
No	19	9.3	(5.4–13.1)	0.249[c]	1.11 (0.97–1.28)	0.128
Yes	35	6.9	(5.0–8.7)			

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease. Values in bold are statistically significant.
[a]Univariate analysis using unpaired *t*-test.
[b]Multivariate analysis using logistic regression with debulking status, tumor grade, and FIGO stage as covariates.
[c]The Welch correction was applied when the S.D. differed significantly between the groups.

variables (debulking status, tumor stage, and histological grade), a tendency was observed for miR-429 expression to associate with recurrence-free survival (HR = 2.10, 95% CI = 0.92–4.79, $P = 0.079$). Similarly, miR-200c and miR-141 also showed this trend (HR = 2.24, 95% CI = 1.00–5.03, $P = 0.051$ and HR = 2.35, 95% CI = 0.98–5.59, $P = 0.054$ respectively) (Supplementary Table 2, see section on supplementary data given at the end of this article). When analyzing OS in the multivariate analysis, the association was not statistically significant for miR-429 or the other microRNAs.

Discussion

Ovarian cancer remains one of the leading causes of cancer death. Most ovarian cancers are detected at advanced stages and, although substantial progress has been made in the treatment of this tumor, lack of response and relapse due to intrinsic or acquired resistance greatly reduce survival rates. Thus, there is a need to improve patient care through the identification of biomarkers predictive of treatment response. This study focuses on the new field of microRNAs, because of their potential to provide novel drug response markers (Yang *et al*. 2008*a*,*b*, Adam *et al*. 2009, Li *et al*. 2009), and gives insight into the role of the miR-200 family in paclitaxel–carboplatin response and survival.

The miR-200 family has been shown to maintain the cellular epithelial phenotype via repression of ZEB1 and ZEB2 and to play an important role in tumor progression (Gregory *et al*. 2008). Interestingly, using *in silico* tools we have found that miR-200b/200c/429 had putative binding sites in the 3′ UTR of the β-tubulin isotypes I, IIa, and III (Supplementary Figure 1, see section on supplementary data given at the end of this article). Since β-tubulin is the therapeutic target of paclitaxel, we speculated that these microRNAs might influence the response of ovarian cancer to paclitaxel-based treatments through the downregulation of these isotypes in the tumoral cells. The miR-200 family of microRNAs has seed sequences differing by one nucleotide (Supplementary Figure 1, see section on supplementary data given at the end of this article) and, although target prediction algorithms assume significant differences in the genes targeted by miR-200b/200c/429 and miR-200a/141, there is evidence indicating a high degree of overlap in target genes (Park *et al*. 2008). This data suggests that multiple members of the miR-200 family may target a large common subset of genes to enhance the efficiency of genetic regulation.

When we measured the expression of the five miR-200 family members in 72 epithelial ovarian cancer samples (Supplementary Figure 3, see section on supplementary data given at the end of this article), we confirmed a high degree of variation (Park *et al*. 2008, Hu *et al*. 2009). The protein expression of classes I, II, and III β-tubulin (Supplementary Figure 2, see section on supplementary data given at the end of this article) showed isotype-specific differences, similar to those reported in a previous study (Ohishi *et al*. 2007). Interestingly, we found classes II and III β-tubulin

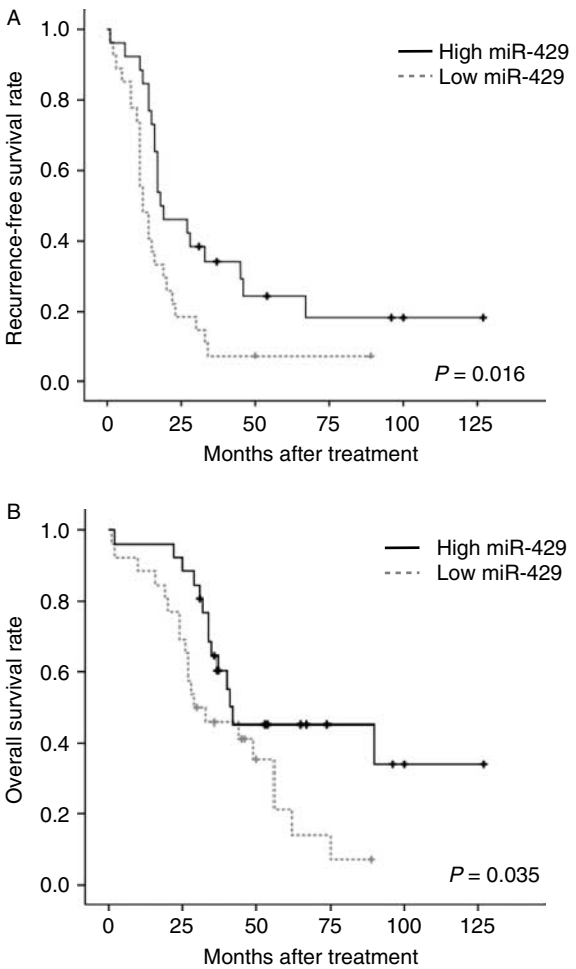

Figure 3 miR-429 is associated with recurrence-free survival and overall survival. The Kaplan–Meier survival analysis evaluating the effect of miR-429 expression on disease outcome for (A) recurrence-free survival and (B) overall survival. The miR-429 median expression was used as cutoff value to divide the patients into low and high miR-429 expressors. The median progression-free survival was 12 months (95% CI 8.2–15.8) for the low and 18 months (95% CI 8.8–27.2) for the high miR-429 expressors. *P* values shown correspond to the log-rank test.

expression to be mutually exclusive, suggesting a complex regulatory mechanism. In other tumor types, isotype III has been detected in 36% of gastric (Urano *et al*. 2006), 40% of head and neck (Koh *et al*. 2009), and 84% of breast (Paradiso *et al*. 2005) cancer samples respectively. β-tubulin III tumoral overexpression has been associated with poor prognosis in a variety of cancer types (Seve *et al*. 2005, Seve & Dumontet 2008, Koh *et al*. 2009), including ovarian carcinomas (Ferrandina *et al*. 2006), although there is one contradictory report (Aoki *et al*. 2009) in clear cell ovarian cancer patients. Concerning response to taxanes, which is closely related to survival rates, high β-tubulin III protein expression was reported to be associated with lack of response in breast (Hasegawa *et al*. 2003, Paradiso *et al*. 2005), lung (Rosell *et al*. 2003, Seve *et al*. 2005), and ovarian (Umezu *et al*. 2008) cancers. In our study, we were not able to detect a significant association between β-tubulin III expression and treatment response, probably due to a small number of high level class III samples. Altogether, these findings seem to reflect an increased resistance of class III to the effect of microtubule-binding drugs (Cochrane *et al*. 2009). In contrast, lack of isotype II expression has been associated with advanced stage and short PFS in ovarian cancer (Ohishi *et al*. 2007). These findings are in agreement with the mutual exclusivity we found for β-tubulins II and III expression.

Interestingly, we found that low levels of miR-200 were associated with high levels of class III protein, implying that β-tubulin III expression could be regulated by this family of microRNAs in clinical samples. In support of this finding, it has been recently shown that the reinstatement of miR-200c in cell lines decreases class III β-tubulin expression and increases sensitivity to microtubule-targeting agents (Cochrane *et al*. 2009) through direct targeting of β-tubulin III (Cochrane *et al*. 2010). However, it should be noted that samples with low levels of miR-200 did not always exhibit high levels of β-tubulin III (Fig. 2). This suggests that in addition to microRNA depletion, other mechanisms, such as epigenetic modifications, are required for β-tubulin III upregulation. We did not find an association between miR-200 and β-tubulins I and II, suggesting that the predicted binding sites for miR-200 in β-tubulins I and IIa genes are either nonfunctional or alternative mechanisms are crucial for the regulation of the eventual protein expression. Mutations on the miRNA-binding sites and alternative cleavage or polyadenylation of the 3′ UTRs are frequent in cancer and might be the mechanism underlying this observation (Blenkiron & Miska 2007, Mayr & Bartel 2009).

Additionally, for class IIa the antibody available for immunohistochemistry detected both IIa and IIb isotypes, which differ in a single nucleotide, and the predicted binding site in IIa was only conserved among mammals. However, for class I the predicted binding site was broadly conserved among vertebrates, similar to that of isotype III.

We then explored a possible role for miR-200 expression as a marker of response to paclitaxel–carboplatin regimen in ovarian carcinomas. Owing to the impact of the cancer stage and histology on response to treatment, we analyzed a homogenous

series of serous adenocarcinomas with FIGO stages III and IV. We found a significant association between miR-200c expression and treatment response: women lacking CR had tumors with significantly lower miR-200c levels than the ones who had achieved CR (HR = 1.43, 95% CI = 1.02–1.99, $P = 0.037$; Table 3); in addition, higher expression of miR-200c was associated with lower relapse/progression rates (HR = 1.17, 95% CI = 1.01–1.34, $P = 0.030$; Table 3). These data seem to indicate that a low miR-200c expression results in high β-tubulin III expression and, thus, increased resistance to paclitaxel-based therapies. *In vitro* studies further support this connection (Cochrane *et al*. 2009, 2010). Regarding prognosis, we found that low tumoral miR-429 was associated with poor PFS and OS (Fig. 3). Multivariate analysis adjusted to relevant clinicopathologic variables revealed a trend for miR-429, miR-200c, and miR-141 with PFS (Supplementary Table 2, see section on supplementary data given at the end of this article), while the association with OS was lost. Hu *et al*. (2009) found a statistically significant association of miR-200a expression with OS and PFS of cancer patients, but Nam *et al*. (2008) described opposite results for the miR-200 family. The discrepancy found by Nam *et al*. could be caused by the small number of samples included in the study (20 serous ovarian carcinoma samples).

Since low tumoral expression of the miR-200 family has been associated with tumor progression and metastasis (Gregory *et al*. 2008, Park *et al*. 2008, Baffa *et al*. 2009), this could lead to a lower OS, independent of treatment response. Our results suggest a possible role for the miR-200 family members as predictive factors for paclitaxel-based response, especially miR-200c, and as prognostic factors in ovarian carcinoma. Because all miR-200 family members share similar targets, but there are differences in the recognition site, we propose that specific members of the family might be more important for prognosis and others for treatment response. In addition, the relative expression levels in the tumor cells could be playing a role in the final regulation of target genes. Thus, the effect of low tumoral miR-200 family expression could be twofold: a decreased response to microtubule-binding drugs and an increased metastasis risk through increased EMT.

Whether the relationship between the expression of miR-200 and first-line treatment response is due to paclitaxel alone or due to combined therapy is unknown, however, this could only be studied using single-agent paclitaxel. Even so, in the context of the new regimens with better response rates (such as combination with antiangiogenic compounds (Burger *et al*. 2007, Cannistra *et al*. 2007, Penson *et al*. 2010)), markers which are able to identify patients who efficiently respond to carboplatin/paclitaxel treatment are relevant. This subset of patients might not benefit from newly targeted drugs, especially if their risk of serious adverse reactions (e.g. bowel perforation) is increased.

In conclusion, we suggest that miR-200 down-regulates β-tubulin III in ovarian tumors. Furthermore, our results suggest a possible role for the miR-200 family both as a prognostic factor and a marker of treatment failure in ovarian carcinoma. Thus, miR-200 might constitute an important biomarker for ovarian cancer patients and could provide the basis for future therapies restoring miR-200 expression in tumor cells. Nevertheless, these data should be further validated in independent cohorts and prospective trials.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1677/ERC-10-0148.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

C Rodríguez-Antona, D Hardisson, M Robledo, and B Martínez-Delgado contributed to the study design; D Hardisson, M Mendiola, J Barriuso, A Redondo, and J de Santiago conceived data collection; S Leskelä, L J Leandro-García, M Mendiola, I Muñoz, and J Barriuso carried out the experiments; S Leskelä, L J Leandro-García, L Inglada, J Barriuso, and C Rodríguez-Antona analyzed the data. All authors were involved in writing the article and had final approval of the submitted and published versions.

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Predictive Biomarkers and Personalized Medicine

Clinical Cancer Research

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 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.

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

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

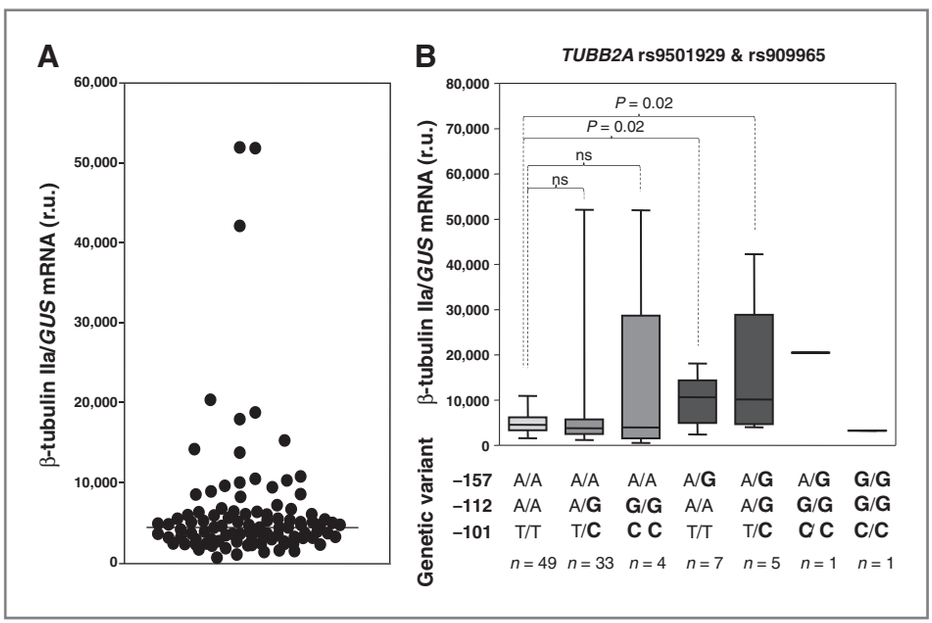

Figure 1. Interindividual variability in *TUBB2A* expression. A, *TUBB2A* mRNA content was measured by quantitative RT-PCR in 100 peripheral blood leukocytes from healthy donors, as described in Materials and Methods. The horizontal bar represents the median value. B, the healthy donors were genotyped 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 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.

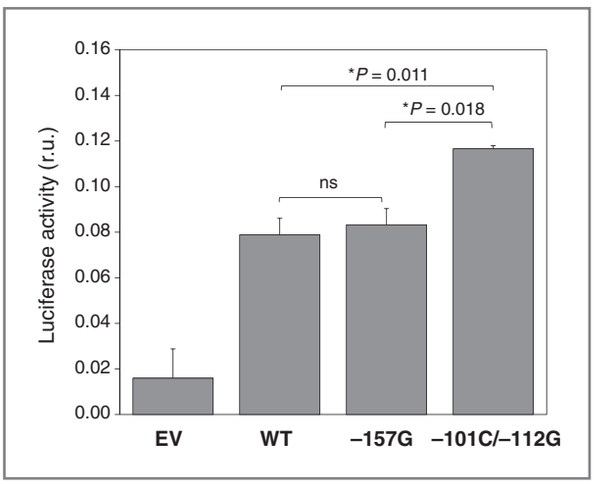

Figure 2. Effect of *TUBB2A* promoter variants on transcriptional activity. H1299 cells were transfected with pGL3-Basic (EV) and luciferase reporter plasmids with different polymorphisms in *TUBB2A* gene: pGL3B_WT (WT), pGL3B_−157G (−157G), and pGL3B_−101C/−112G (−101C/−112G). The cells were cotransfected 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.

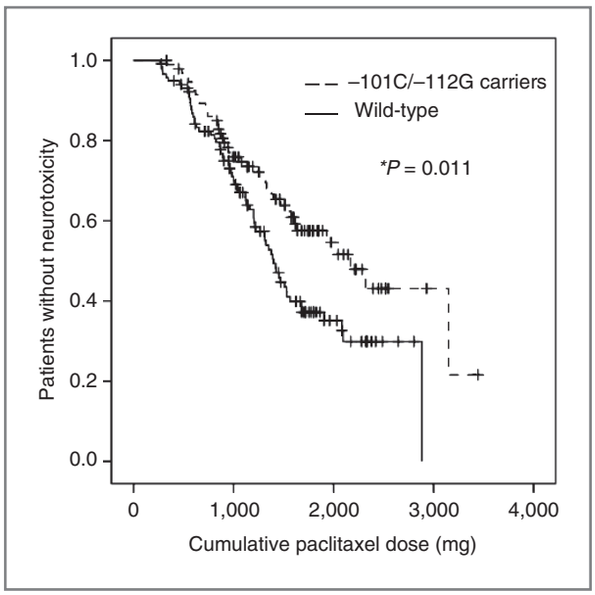

Figure 3. Kaplan–Meier analysis of cumulative dose of paclitaxel up to the development of grade 2 sensory peripheral neuropathy, according to −101C/−112G variants in *TUBB2A*. Patients treated with paclitaxel were grouped according to *TUBB2A* genotype. Those carrying 1 or 2 variant alleles had a significantly lower risk of paclitaxel-induced neurotoxicity. The *P* value shown corresponds to univariable log-rank test.

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

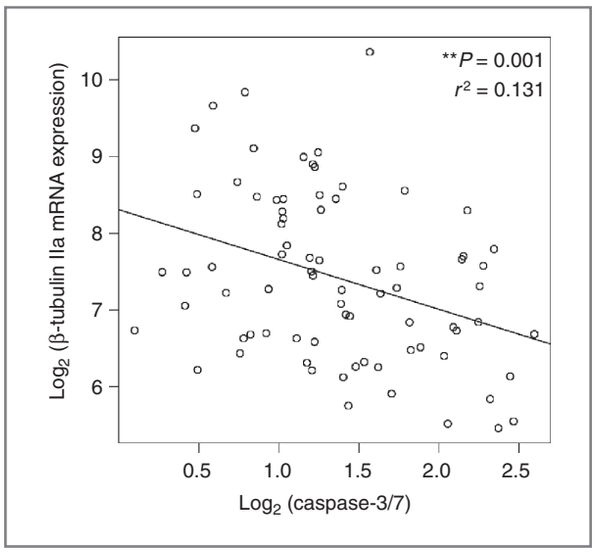

Figure 4. Inverse correlation between *TUBB2A* gene expression and paclitaxel-induced apoptosis. Lymphoblastic cell lines (CEU, $n = 77$) were treated with paclitaxel to measure caspase-3/7 activation (apoptosis), and Affymetrix exon array was used to measure *TUBB2A* expression in the same cell lines, 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Cancer Research

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Hematologic β-Tubulin VI Isoform Exhibits Genetic Variability That Influences Paclitaxel Toxicity

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Abstract

Cellular microtubules composed of α-β-tubulin heterodimers that are essential for cell shape, division, and intracellular transport are valid targets for anticancer therapy. However, not all the conserved but differentially expressed members of the β-tubulin gene superfamily have been investigated for their role in these settings. In this study, we examined roles for the hematologic isoform β-tubulin VI and functional genetic variants in the gene. β-tubulin VI was highly expressed in blood cells with a substantial interindividual variability (seven-fold variation in mRNA). We characterized DNA missense variations leading to Q43P, T274M, and R307H, and a rare nonsense variant, Y55X. Because variations in the hematologic target of microtubule-binding drugs might alter their myelosuppressive action, we tested their effect in cell lines stably expressing the different β-tubulin VI full-length variants, finding that the T274M change significantly decreased sensitivity to paclitaxel-induced tubulin polymerization. Furthermore, patients treated with paclitaxel and carrying β-tubulin VI T274M exhibited a significantly lower thrombocytopenia than wild-type homozygous patients ($P = 0.031$). Together, our findings define β-tubulin VI as a hematologic isotype with significant genetic variation in humans that may affect the myelosuppresive action of microtubule-binding drugs. A polymorphism found in a tubulin isoform expressed only in hemapoietic cells may contribute to the patient variation in myelosuppression that occurs after treatment with microtubule-binding drugs. *Cancer Res; 72(18); 1–9. ©2012 AACR.*

Introduction

Microtubules are ubiquitous and highly dynamic polymers of α-β-tubulin heterodimers indispensable for a variety of cellular functions such as structure maintenance, intracellular transport, cell signaling, migration, and mitosis. Several of the most common chemotherapeutic drugs, such as taxanes, vinca-alkaloids, and epothilones, base their mechanism of action on binding to microtubules and altering their dynamics, which leads to mitosis arrest and cell death (1, 2). The therapeutic target of these drugs is β-tubulin, which consists of 8 isotypes encoded by multiple genes that exhibit a tissue-specific expression. In a previous study, we showed that isotypes I, IVb, and V are constitutive, isotypes IIa, IIb, III, and IVa are neuronal, and isotype VI is hematopoietic cell-specific and was detected in platelets, lymphocytes, bone marrow, and spleen (3, 4). The β-tubulin isotypes are highly conserved and have similar sequences, however, the C-terminal region exhibits higher variability and seems to confer differences in microtubule polymerization and stability. The fact that rare mutations in β-tubulin IIb and III lead to a spectrum of severe neuronal disorders suggests specific functions that cannot be compensated by alternative isotypes (5, 6). β-Tubulin VI knockout mice studies proved a specialized role for this protein in platelet synthesis, structure, and function (7, 8).

Because of the crucial role of β-tubulin in cells, genetic variation is not allowed. However, the hematologic isotype VI is an exception, and a common missense polymorphism (Q43P) has been associated with an altered risk of cardiovascular disease by modulating platelet function and structure (9, 10). There is also a report on a rare β-tubulin VI missense variant (R318W) responsible for congenital macrothrombocytopenia (11). Other genetic variations in β-tubulin isotypes correspond to cell lines and lead to acquired resistance to microtubule-binding drugs (12–14). Variations in β-tubulin isotype composition in tumors have also been associated with decreased sensitivity to these drugs (15–18). Thus, polymorphisms in β-tubulin VI, leading to a variant protein or altered expression,

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/)

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might result in differences in the effect of β-tubulin–binding drugs among patients. Because of the hematologic role of β-tubulin VI, these polymorphisms could affect the action of the drugs in blood cells, leading to interpatient differences in myelosupression. However, at the moment, the expression of β-tubulin VI has not been well-established, and polymorphisms in this gene have not been investigated.

In this study, we characterized β-tubulin VI expression in hematologic cells and screened for genetic variation by sequencing the coding region of the β-tubulin VI gene (*TUBB1*). We generated cell lines stably expressing the variant β-tubulin VI proteins detected and conducted functional assays. Furthermore, the clinical implications of these polymorphisms were studied in patients with hematologic malignancies treated with paclitaxel.

Materials and Methods

Human samples and patients

cDNAs from 7 different blood fractions were obtained from BD Biosciences (Human Blood Fractions MTC Panel), peripheral blood lymphocytes, platelets, and neutrophils were isolated from healthy volunteers, and $CD34^-$ bone marrow cells were isolated from bone marrow aspiration following previously described procedures (19). Frozen samples from peripheral T-cell lymphomas were collected through the Spanish National Cancer Centre (Madrid, Spain) tumor bank network from pathology departments of different hospitals in Spain (20). The main characteristics of this human material and cell lines used for mRNA quantification are shown in Supplementary Table S1. For the estimation of allele and haplotype frequencies, we used DNA isolated from unrelated individuals from different ethnic groups, specifically, 481 Caucasians from Spain, 106 Asians from China, and 71 Africans from Tanzania.

Forty-nine patients with hematologic malignancies planned for autologous transplant (20 with lymphoma, 15 with multiple myeloma, and 14 with acute leukemia), who underwent mobilization of hematopoietic progenitor cells with paclitaxel 170 mg/m^2 i.v. by continuous infusion for 24 hours followed by 8 μg/kg s.c. recombinant human granulocyte colony-stimulating factor (rhG-CSF) daily, and that had leukapheresis product available, were included in the study (21). In this series, recruited between 1999 to 2008, the time elapsed from the last cytotoxic treatment was at least 3 weeks, to allow recovery of peripheral blood counts before paclitaxel administration. Peripheral blood counts were assessed on days 5, 7, and daily afterward. Maximal hematologic toxicity was retrospectively assessed and recorded according to the National Cancer Institute-Common Toxicity Criteria version 3. The characteristics of the patients, including age at treatment, previous treatments, state of disease at stem cell harvest, weeks from the last treatment, and baseline platelet counts at paclitaxel treatment initiation are provided in Supplementary Table S3.

RNA isolation and quantitative RT-PCR

RNA was extracted from blood cells, cell lines, and frozen tumoral tissue using TRI-reagent (Molecular Research Center Inc.) and the concentration quantified by using Nanodrop ND-1000 (Wilmington). One microgram of total RNA was reverse transcribed using Superscript II (Invitrogen) and an oligo dT14 primer following the manufacturer's instructions. The mRNA content of the different β-tubulin isotypes was quantified by quantitative reverse transcription-PCR (qRT-PCR) with the Sequence Detection System 7900HT (Applied Biosystems) using conditions, primers, and probes previously described (4). Normalization was carried out with the internal standard β-glucuronidase (*GUS*). Negative controls were present in all PCR series and assays were carried out in triplicates. The $\Delta\Delta C_t$ method (22) was used for the calculation of mRNA content.

DNA isolation, sequencing, and genotyping

Genomic DNA was isolated from the peripheral blood lymphocytes of the healthy volunteers and from the leukapheresis product of the hematologic patients using FlexiGene DNA Kit (QIAGEN), and the DNA concentration was determined using PicoGreen dsDNA quantification reagent (Invitrogen). For *TUBB1* sequencing, the exons and proximal promoter region were amplified by PCR using specific primers designed to avoid cross-reactions with other β-tubulin isotype genes/pseudogenes (Supplementary Table S2). 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 the *TUBB1* coding polymorphisms 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.

Platelet activation and aggregation assays

The effect of β-tubulin VI R307H polymorphism in platelet function was investigated by conducting aggregation assays in 6 healthy volunteers of known genotype (3 women homozygous for the wild-type allele and 2 women and 1 man homozygous for the variant allele). Platelet aggregation was measured in nonadjusted-citrated platelet-rich plasma obtained by centrifuging blood at 140 × *g* for 12 minutes. Platelets were stimulated with 6-mer thrombin receptor agonist at 0.78 to 25 μmol/L (Sigma-Aldrich), collagen at 0.125 to 2 μg/mL (Menarini Diagnostics), ADP at 0.16 to 0.5 μmol/L (Menarini Diagnostics), arachidonic acid at 0.4 to 1.6 mmol/L (Bio-Rad), and ristocetin at 0.75 to 1.25 mg/mL (Sigma-Aldrich). Changes in light transmission of platelet-rich plasma over baseline were recorded for 5 minutes using an Aggrecorder II aggregometer (Menarini Diagnostics).

Generation of cell lines expressing β-tubulin VI wild-type and variant proteins

We amplified the full coding sequence of *TUBB1* using specific primers that introduced *Nhe*I and *Not*I cleavage sites (Supplementary Table S2) and cDNA from peripheral blood lymphocytes that carried in homozygosity the wild-type *TUBB1* coding region. The PCR product was cloned into pIRESpuro2 vector (Clontech) to generate pIRESpuro2_TUBB1 wild-type

plasmid. By means of the GeneTailor Site-Directed Mutagenesis System (Invitrogen) and following the manufacturer's indications and adequate primer pairs (Supplementary Table S2), the pIRESpuro2_TUBB1 wild-type construct was used to generate plasmids that contained *TUBB1* sequence with variants encoding for the following protein changes: Q43P, Y55X, T274M, R307H, and T274M/R307H. Correct *TUBB1* sequence and lack of artifacts was confirmed by sequencing.

MCF7 breast cancer cells were provided by Dr M. Esteller (IDIBELL, Barcelona, Spain). MCF7 cells were chosen for heterologous expression of β-tubulin VI based on their lack of endogenous expression of this protein and sensitivity to microtubule-binding drugs. MCF7 cells were transiently transfected with the different *TUBB1* constructs and the empty vector by using Lipofectamine-2000 (Invitrogen) following the manufacturer's instructions. To generate stable cell lines, the different *TUBB1* constructs and the empty vector were electroporated using the Gene Pulser XCell Electroporation System (Bio-Rad) into cells and 24 hours later, 0.5 μg/mL of puromycin (Sigma-Aldrich) was added for selection. Puromycin-resistant clones were analyzed for β-tubulin VI expression by Western blot analysis. Control cells were also selected with puromycin, all resistant empty vector clones pooled together. Stable transfectants were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS, penicillin, streptomycin, and puromycin at a concentration of 0.4 μg/mL.

β-Tubulin VI protein detection

For Western blotting, total cell extracts from cell lines were separated by using the Criterion XT Gels Bis-Tris 10% and the Criterion electrophoresis cell (Bio-Rad) and transferred to polyvinylidene fluoride membranes (Immobilon-P Membrane, Millipore) using the Trans Blot Semi-Dry Blotter (Bio-Rad). Protein concentration was measured by using Bio-Rad Protein Assay (Bio-Rad), and equal loading of proteins was verified by Ponceau S staining. The membranes were blocked and then incubated with a rabbit polyclonal antibody specific for human β-tubulin VI (from Dr. Paraskevi Giannakakou; ref. 3), a mouse antibody recognizing all β-tubulin isotypes (clone 2.1, Sigma; 1:1,000 dilution), or a rat anti-α–tubulin (clone YL1/2; Chemicon International; 1:1,000 dilution). After washing, the membranes were incubated with the corresponding secondary antibody: goat anti-rabbit (Alexa Fluor 680 nm, Molecular Probes; 1:15,000 dilution), goat anti-mouse (DyLight 800, Thermo Scientific; 1:10,000 dilution), or donkey anti-rat (IRdye 800 nm, Rockland Immunochemicals; 1:15,000 dilution). The fluorescence signal was detected using the Odyssey infrared imaging system (LI-COR).

Immunofluorescence microscopy was conducted as previously described (23). In brief, cells were cultivated on coverslips overnight and fixed the next day with 4% paraformaldehyde, permeabilized with Triton-X-100 0.5% in TBS, and blocked with 10% goat serum. The same primary antibodies as those described for Western blotting were used. Specific secondary antibodies, Alexa-rabbit-405 nm and Alexa-rat 568 nm, were used. Cells were imaged using a Zeiss LSM 5 LIVE confocal microscope using a ×40/1.3 EC Plan Neofluar objective, a ×63/1.4 Plan APOCHROMAT objective, and ×100/1.4 Plan APOCHROMAT objectives. All images were acquired and analyzed using Zeiss LSM 5 LIVE software.

Tubulin polymerization assay

Quantitative drug-induced tubulin polymerization assays were conducted as previously described (12). In brief, cells were grown in 24-well plates overnight and incubated for 6 hours with paclitaxel at concentrations of 0, 10, 100, and 1,000 nmol/L. The cells were washed twice with PBS before lysis at 37°C for 5 minutes in the dark with 100 μL of hypotonic buffer (20 mmol/L Tris-HCl pH 6.8, 1 mmol/L $MgCl_2$, 2 mmol/L EGTA, and 0.5% Nonidet P40) containing protease inhibitors (Protease Cocktail inhibitor tablets; Roche). The lysates were transferred to 1.5 mL Eppendorf tubes, rinsed with 100 μL of hypotonic buffer, mixed by vortexing, and centrifuged at 14,000 rpm for 10 minutes at room temperature. The 200 μL supernatants containing soluble (cytosolic) tubulin were transferred to another tube, and pellets containing polymerized (cytoskeletal) tubulin were resuspended in 200 μL of hypotonic buffer. The cytosolic and cytoskeletal fractions were each mixed with electrophoresis sample buffer, sonicated, boiled, and analyzed by immunoblotting. The percentage of polymerized tubulin was determined by dividing the densitometry value of polymerized tubulin by the total tubulin content (the sum of the densitometry values of soluble and polymerized tubulin) in at least 3 independent experiments.

Statistical analysis

The myelotoxicity of the patients was analyzed taking into account gender, age at paclitaxel treatment, type of disease, previous treatments, state of disease at stem cell harvest, weeks from last treatment, and baseline platelet counts at the initiation of paclitaxel and genotypes. For the analysis, quantitative variables were dichotomized according to the median. To include previous chemotherapy as a covariable, we used a bone marrow toxicity scoring system, which takes into account drugs and number of chemotherapy cycles used (24). This chemotherapy score correlates with damage to the stem cell pool caused by previous chemotherapy and with the ability to mobilize hematopoietic cells (25, 26). Acute leukemias were excluded from this analysis because, although treatments only include low/medium myelotoxic drugs and they have low chemotherapy score, the intense dosages in these protocols cause prolonged aplasias (Supplementary Table S3). The χ^2 test and Fisher exact test were used to compare the hematologic toxicity grade of patients with clinical variables and genotype. To correct for the possible effect of previous treatments on the thrombocytopenia, the Mantel–Haenszel test was used. Nominal 2-sided P values < 0.05 were considered statistically significant. All statistical analyses were carried out using SPSS software package version 17.0 (SPSS).

Results

β-Tubulin isotype VI has a broad and variable expression in blood cells

We previously showed that β-tubulin VI expression was exclusively found in hematopoietic lineage cells, specifically, in

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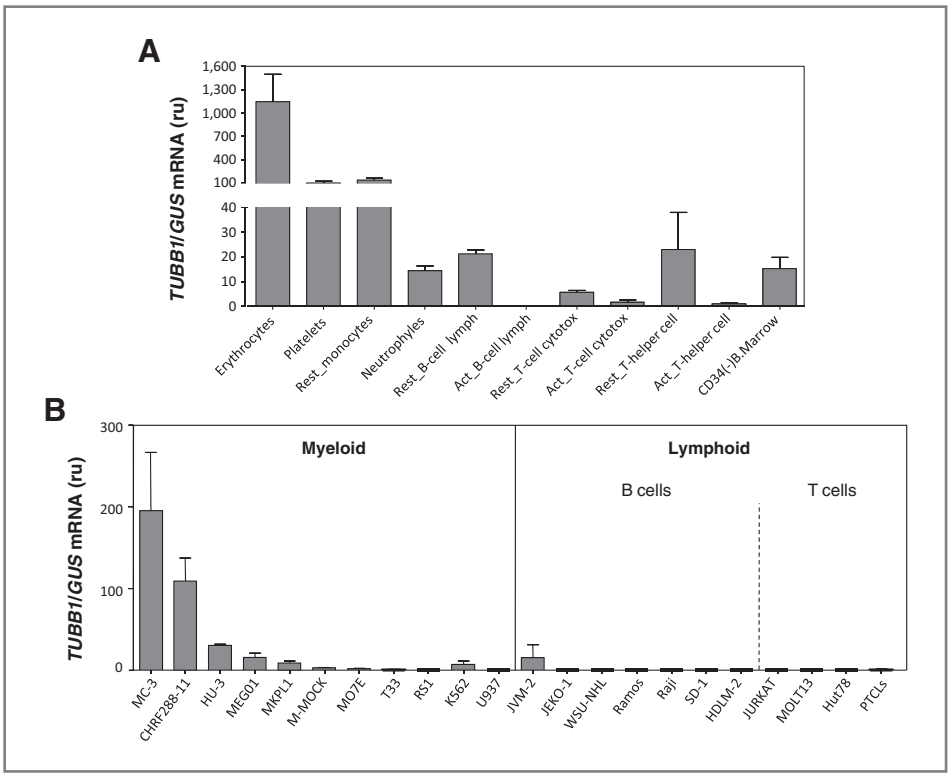

Figure 1. β-tubulin VI mRNA content in hematologic cells. qRT-PCR was used to determine β-tubulin VI (*TUBB1*) mRNA quantities in different cells. A and B, *TUBB1* mRNA content in different nontumoral human hematologic cells (A) and in different cell lines and tumors of myeloid and lymphoid origin (B). mRNA content is compared with platelets expression (100%). ru, relative units; Rest, resting; Act, activated.

platelets, lymphocytes, bone marrow, and spleen; however, a detailed characterization of β-tubulin VI expression has not been conducted. Here, we show that β-tubulin VI mRNA was detected in all blood samples tested with the highest expression corresponding to erythrocytes, platelets, and monocytes (cells of myeloid origin; Fig. 1A). To further investigate the expression of β-tubulin VI, we also determined β-tubulin VI mRNA content in hematologic malignancies by studying tumoral cell lines and samples. Again, cells of myeloid origin showed higher expression than those of lymphoid origin, with the megakaryocytic MC-3 and CHRF288-11 cell lines showing the highest expression levels (Fig. 1B).

To confirm the relevance of β-tubulin VI in blood cells, we studied its expression in peripheral blood lymphocytes from 100 healthy volunteers. Figure 2A shows that isotype VI was the major β-tubulin component of lymphocyte microtubules, followed by β-tubulins IVb and I, accounting for 82, 10, and 7.5% of the total β-tubulin content, respectively. Interestingly, β-tubulin VI mRNA content was subjected to a substantial interindividual variability (7.2-fold variation in expression; Fig. 2B). To substantiate this variability, we also measured the expression of the ubiquitous and highly conserved β-tubulins IVb and I in the same samples, finding a 2.5- and 2.2-fold variation in mRNA content, respectively (data not shown).

TUBB1 gene has common missense polymorphisms

To determine whether *TUBB1* gene could differentiate from the other β-tubulin isotypes and be subjected to common genetic variability, we sequenced the 4 exons and part of the 5′ and 3′ untranslated region of *TUBB1* in genomic DNA from 20 unrelated Caucasians. In addition to the already studied missense polymorphisms, rs463312 (Q43P) and rs415064 (Q43H), we found 2 other nucleotide changes leading to amino acid changes, rs35565630 (T274M) and rs6070697 (R307H), and one resulting in a premature stop codon (c.585G>A; Y55X). To determine the allelic frequency of these genetic variations, we conducted genotyping in a series of unrelated Caucasians, Asians, and Africans (Table 1). The nucleotide change leading to an early stop codon was only detected in the initial case used for sequencing. Regarding the other variants, the polymorphism leading to R307H was the most frequent, followed by those corresponding to Q43P, Q43H, and T274M, this latter not detected in Asians. When we established the haplotypes formed by these missense variants (Table 1), we found that, as previously observed, rs463312 and rs415064 were in total linkage disequilibrium resulting exclusively in Q43P. rs35565630 was found only in the presence of rs6070697, thus, encoding a protein with 2 amino acid changes (T274M and R307H), whereas a relatively high number of cases carried only rs6070697 (R307H).

Concerning the putative effect of the *TUBB1* coding variants, previous results have shown that Q43P affects cardiovascular risk through an alteration of platelet aggregation. To determine whether the newly described missense polymorphisms could also alter platelets function, we isolated DNA from 100 healthy volunteers and identified individuals carrying the variants in homozygosity. For T274M, we could not find homozygous variant individuals, thus, we conducted platelet activation and aggregation assays only for rs6070697 (R307H) and wild type homozygous individuals; however, we did not detect significant differences (data not shown). In addition, we tested

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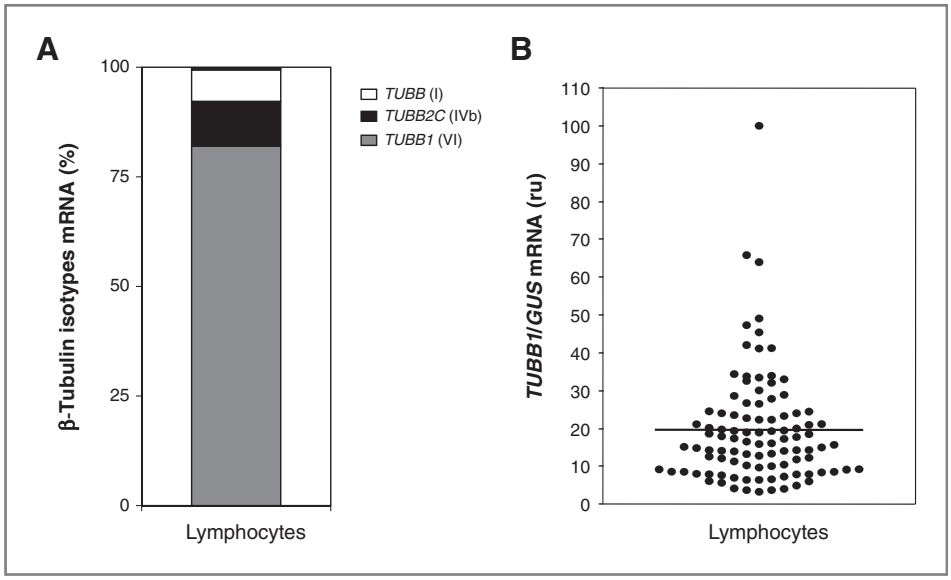

Figure 2. β-tubulin isotypes relative expression in lymphocytes and interindividual variability in β-tubulin VI mRNA expression. A, β-tubulin isotypes mean relative mRNA expression in peripheral blood lymphocytes from 100 healthy volunteers (*TUBB1*: 82.00%; *TUBB2C*: 10.00%; *TUBB:* 7.10%; *TUBB6:* 0.35% and *TUBB2A:* 0.27%. mRNA from *TUBB3, TUBB2B*, and *TUBB4* below detection limits). B, *TUBB1* mRNA content was quantified by qRT-PCR in peripheral blood lymphocytes isolated from 100 healthy volunteers. ru, relative units.

whether the missense polymorphisms could be associated with an altered *TUBB1* mRNA content and thus explain the inter-individual variation found in the expression of this gene (Fig. 2B); however, none of the polymorphisms was significantly associated with an altered mRNA content (data not shown). Sequencing of *TUBB1* proximal promoter region in individuals with maximal and minimal mRNA content did not identify polymorphisms associated with the expression of the gene.

β-Tubulin VI T274M alters paclitaxel effect on tubulin polymerization and is associated with decreased thrombocytopenia in patients treated with paclitaxel

To test the effect of the *TUBB1* missense polymorphisms, we carried out transient transfections using appropriated expression vectors in MCF7 cells. These cells are derived from a solid tumor and do not express β-tubulin VI protein. As shown in Fig. 3A and B, the exogenously expressed β-tubulin VI proteins were correctly produced and colocalized with α-tubulin, indicating a correct incorporation into the cytoskeleton of the transfected cells. Cells stably expressing the different β-tubulin VI proteins were used to study tubulin polymerization after treatment with increasing amounts of paclitaxel. We found that in control cells, transfected with the empty vector, most of the cellular β-tubulin shifted to a polymerized form at 100 nmol/L paclitaxel. This same effect was also appreciated in cells expressing wild-type, Q43P, and R307H β-tubulin VI proteins. However, cells expressing T274M and T274M/R307H variant proteins had significantly higher amounts of soluble

Table 1. Genetic variants in *TUBB1* coding region and common haplotypes

Variants in coding region	Amino acid change	Caucasians ($N = 481$)		Asians ($n = 106$)		Africans ($n = 71$)	
		Frequency[a]	n[b]	Frequency	n	Frequency	n
rs463312	Q43P	0.042	130	0.086	105	0.029	68
rs415064	Q43H	0.042	130	0.086	105	0.029	68
—	Y55X	0.001	451	0.000	106	0.000	71
rs35565630	T274M	0.036	481	0.000	105	0.014	71
rs6070697	R307H	0.192	453	0.107	98	0.123	69
Haplotypes[c]	**Amino acid changes**	**Frequency**		**Frequency**		**Frequency**	
Hap_AGCCG	None	0.791		0.823		0.846	
Hap_AGCCA	R307H	0.146		0.107		0.110	
Hap_CCCCG	Q43P	0.037		0.069		0.029	
Hap_AGCTA	T274M/R307H	0.023		0.000		0.013	

[a]Frequency for the different alleles and haplotypes.
[b]Number of individuals used in each determination.
[c]Haplotypes estimated with frequencies >1%. The nucleotides correspond to rs463312, rs415064, the nucleotide change leading to Y55X, rs35565630, and rs6070697.

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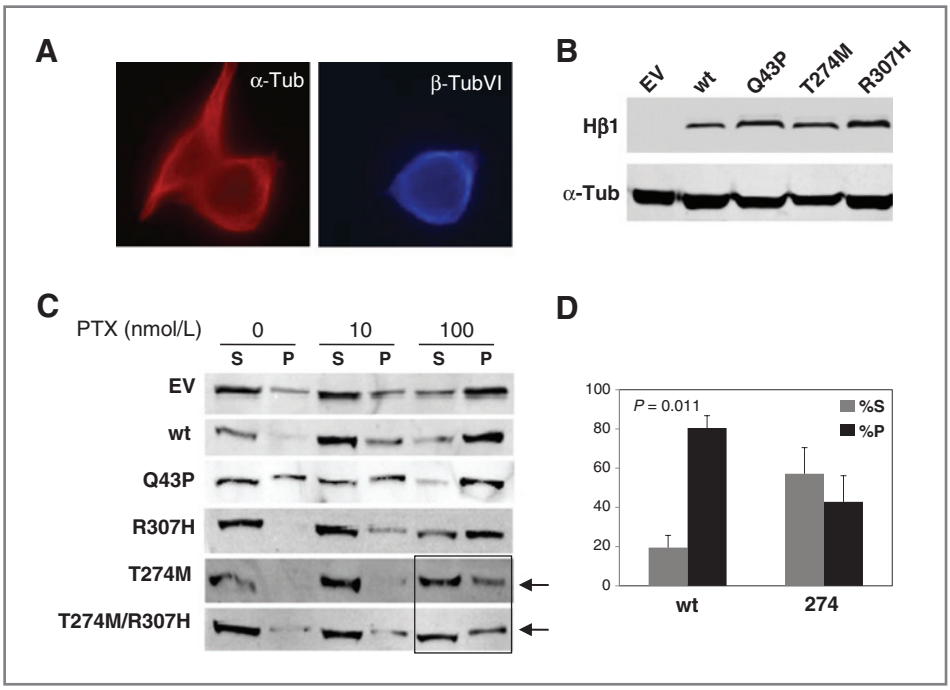

Figure 3. Cells expressing β-tubulin VI T274M variant protein are less sensitive to the effect of paclitaxel on tubulin polymerization. A, confocal microscopy images of β-tubulin VI transient transfection in MCF7 cells. α-Tubulin and β-tubulin VI staining are visualized in red (568 nm) and blue (405 nm), respectively. B, β-tubulin VI protein detection by Western blot analysis in MCF7 cells stably expressing the different variants and those transfected with the empty vector (EV). C, MCF7 cells stably expressing the different β-tubulin VI variants were treated with paclitaxel (PTX) for 6 hours. Control- and paclitaxel-treated samples were lysed and the polymerized (P) and the soluble (S) tubulin fractions were separated by centrifugation, loaded on adjacent lanes in SDS-PAGE, and immunoblotted with an antibody against total β-tubulin. No statistically significant differences were found for any of the variant proteins, except for those with T274M. Arrows indicate the lanes with significant differences. D, the percentage of polymerized (%P) and soluble (%S) tubulin was determined by dividing the densitometric value of polymerized (P) and soluble (S) tubulin by the total tubulin content (P + S). The bars in the graph represent the mean values of 3 different experiments.

β-tubulin at 100 nmol/L paclitaxel ($P = 0.011$; see Fig. 3C and D). These results suggest that β-tubulin VI T274M variant protein is less sensitive to the polymerization effect of paclitaxel.

Because β-tubulin VI is a hematologic isotype, we investigated whether β-tubulin VI T274M patients could be less sensitive to the myelosuppressive effect of paclitaxel. To determine this, we genotyped a series of 49 patients with nonsolid tumors scheduled for autologous transplant treated for mobilization of hematopoietic progenitor cells with paclitaxel 170 mg/m^2 i.v. by continuous infusion for 24 hours, followed by 8 μg/kg s.c. rhG-CSF daily. With this highly myelosupressive protocol, grades III and IV leukocytopenia and thrombocytopenia occurred in 55% and 43% of patients, respectively. We did not find a statistically significant association between clinical variables (e.g. type of disease, previous treatments, state of disease at stem cell harvest, weeks from last treatment, and platelet counts at baseline before the initiation of treatment with paclitaxel) and the hematologic toxicity of the patients; however, we found a statistically significant effect for T274M polymorphism. Heterozygous T274M patients showed significantly lower thrombocytopenia than homozygous wild-type subjects. The 2 T274M patients exhibited grade I toxicity, whereas 85% of homozygous wild-type patients developed higher thrombocytopenia grades (II, III, or IV; $P = 0.031$; Fig. 4). The association was also significant (Mantel–Haenszel test, $P = 0.028$) when the previous chemotherapy treatments were taken into account. No significant association was found between T274M and neutropenia. We also genotyped the patients for Q43P and R307H variants and, as expected, no differences in thrombocytopenia and neutropenia were found (data not shown).

Discussion

In this study, we show that β-tubulin VI is a hematology-specific isotype subjected to a substantial genetic difference that is likely contributing to the interindividual variability observed in the myelosuppressive effect of paclitaxel. A thorough characterization of β-tubulin VI expression in cells of hematopoietic lineage revealed a high β-tubulin VI expression not only in lymphocytes and platelets, as previously described (3, 4), but also in most blood cells and several hematologic malignancies (Fig. 1). These data and the lack of β-tubulin VI in nonhematopoietic tissues (4) indicate that this is a hematology-specific isotype, and hence a major target mediating the hematologic toxicity of the β-tubulin–binding drugs. In contrast, the low expression of this isotype in hematologic malignancies of lymphoid origin does not support a major role of the β-tubulin VI in the physiopathology of these diseases. Furthermore, we found substantial interindividual variability in

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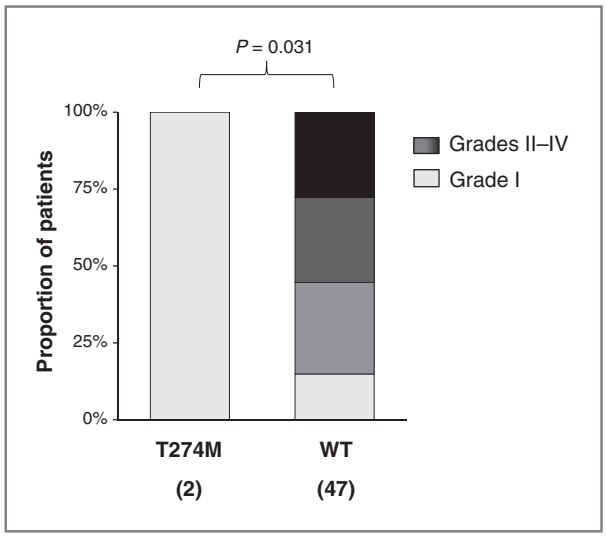

Figure 4. Patients that are carriers of β-tubulin VI T274M polymorphism develop less thrombocytopenia after paclitaxel treatment. Patients treated with paclitaxel 170 mg/m^2 i.v. by continuous infusion for 24 hours were genotyped and the thrombocytopenia grade developed after paclitaxel treatment (grade I vs. grades II, III, or IV) was compared with the homozygous wild-type patients using Fisher exact test.

β-tubulin VI mRNA content and common amino acid changes affecting *TUBB1* gene (Fig. 2 and Table 1).

So far, β-tubulin isotype genes have been considered highly conserved (27); however, here we prove that β-tubulin VI differentiates in this respect from the rest of isotypes and exhibits common variation in the coding region. β-tubulin VI Q43P was previously described and associated with a reduced risk of arterial thrombosis and an increased risk of intracerebral hemorrhage by modulating the platelet function and structure (9, 10). In addition, a rare β-tubulin VI variant, R318W, was described in a case of congenital macrothrombocytopenia (11). In this study, a nonsense variant (Y55X) was detected in heterozygosity; however, no deviations from normal ranges were found in the hemograms of this individual. So far, all the β-tubulin pathogenic variants are missense mutations (5, 6, 11), suggesting that early truncated proteins, that do not integrate into microtubules, might not be as damaging as variant proteins that lead to altered dynamic instability of microtubules (5, 6). This is in agreement with the lack of Y55X phenotype. The R307H polymorphism is located in a conserved region encoding β-tubulin VI M-loop; however, computational algorithms (PolyPhen and SIFT) predicted a benign variant and we found no significant effects on platelet activation and aggregation. T274M change also affects a conserved amino acid in the M-loop and was predicted as probably damaging by PolyPhen and SIFT; however, we detect this variant in the general population, suggesting it will not substantially alter its function. In regard to the microtubule-targeted drugs, residue 274 is located near the taxanes binding site (28).

We found that cells stably expressing β-tubulin VI 274M variant, alone or in combination with 307H, were less sensitive to the polymerizing effect of paclitaxel compared with control cells transfected with the empty vector and those expressing β-tubulin VI wild-type, 43P, and 307H variant proteins (Fig. 3). Human β-tubulin isotypes are essentially invariant within paclitaxel-binding site and, specifically, residue 274 is evolutionarily conserved in all vertebrate β-tubulins and all known β-tubulin isotypes in these organisms (29). Interestingly, residue 274 clusters in a space that has been identified as essential for interaction of paclitaxel with tubulin and an acquired mutation in residue 274 of β-tubulin I (T274I) renders cell lines resistant to paclitaxel and epothilone A (28, 30). These data suggest that variants affecting threonine 274 can lead to impaired drug binding and, thus, patients carrying the T274M variant could be resistant to the myelosuppressive effects of these microtubule-binding drugs. To investigate the impact of this polymorphism in patients and its potential as a marker of hematologic toxicity, we worked with a series of patients treated with paclitaxel. Because paclitaxel hematologic toxicity greatly depends on the time used for drug infusion (31), we decided to work with an outstanding set of 49 patients treated with a highly myelosuppressive protocol used for mobilization of hematopoietic progenitor cells. Although only 2 patients were T274M carriers, they showed a statistically significant lower grade of thrombocytopenia ($P = 0.031$; Fig. 4), suggesting that *TUBB1* T274M conferred protection against this paclitaxel toxicity. It is important to highlight that the T274M carriers had similar characteristics than the noncarriers in terms of age, disease state, base line platelets, and chemotherapy score (Supplementary Table S3). With respect to the type of disease, both were patients with myeloma with treatments that have an important effect on platelet production. Concerning the interval from last chemotherapy, the two carriers were among the patients with shorter times. No significant associations were found for leukocytopenia, but the results are inconclusive, because patients were treated with granulocyte colony-stimulating factor (G-CSF) to stimulate the production of neutrophils, and this is an important confounding factor. The effect found for T274M in thrombocytopenia risk is supported by the critical role that β-tubulin VI plays in platelets, showed both in knockout mice (7) and in patients carrying a mutation in this gene (11). Although in these previous studies, no pathogenic phenotypes were reported in neutrophils, we detected high contents of β-tubulin VI in these cells (Fig. 1A). This, together with the fact that the microtubule-binding agent 2-methoxyestradiol does not have an effect on β-tubulin VI (3) and does not result in any type of myelosupression (32), supports a function of this isotype also in neutrophils and consequently in drug induced neutropenia. The similar structure of paclitaxel and docetaxel, which share the binding domain with epothilones, and the fact that cell lines expressing β-tubulin I T274I are resistant to all of these agents (28), warrants further studies to investigate the role of T274M in the hematologic toxicity of these drugs. It can be estimated that approximately 7% of Caucasians carry T274M and might show differences in myelosupression.

In summary, this is the first study showing that β-tubulin VI is a hematology-specific isotype that differentiates from other β-tubulin genes by relevant genetic and expression variability. In addition, we show that β-tubulin VI T274M decreases the effect of paclitaxel on tubulin polymerization and, although

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validation in independent patient series is required, we provide data supporting that it might constitute a marker of hematologic toxicity induced by paclitaxel.

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, R. Torres, M.L. Lozano, A. Cascon
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L.J. Leandro-Garcia, S. Leskela, L. Inglada-Perez, J. Rivera, M.L. Lozano, C. Rodriguez-Antona
Writing, review, and/or revision of the manuscript: L.J. Leandro-Garcia, S. Leskela, L. Inglada-Perez, J.C. Ramírez, S. Alvarez, J. Rivera, C. Martinez, M.L. Lozano, A. Cascon, M. Robledo, C. Rodriguez-Antona
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L.J. Leandro-Garcia, S. Leskela, S. Alvarez, M. Robledo
Study supervision: L.J. Leandro-Garcia, L. Inglada-Perez, M. Robledo, C. Rodriguez-Antona

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