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Pharmacogenetics of taxanes: Identification and characterization of molecular mechanisms underlying toxicity and lack of response

Doctoral Thesis

SUSANNA LESKELÄ

CENTRO NACIONAL DE INVESTIGACIONES ONCOLÓGICAS

Programa de Genética del Cáncer Humano

Grupo de Cáncer Endocrino Hereditario

Directoras de Tesis

Dra. Cristina Rodríguez González de Antona

Dra. Mercedes Robledo Batanero

cnio
Centro Nacional
de Investigaciones
Oncológicas

Dra. Mercedes Robledo Batanero, Jefe del Grupo de Cáncer Endocrino Hereditario del Centro Nacional de Investigaciones Oncológicas (CNIO), como Profesora Honoraria del Departamento de Biología Molecular de la Universidad Autónoma de Madrid y como Co-Directora y Tutora,

CERTIFICA:

Que Doña Marja Susanna Leskelä, Licenciada en Biología por la Universidad de Helsinki, ha realizado la presente Tesis Doctoral: "**Pharmacogenetics of taxanes: Identification and characterization of molecular mechanisms underlying toxicity and lack of response**" y que a mi juicio reúne plenamente todos los requisitos necesarios para optar al **Grado de Doctor en Biología**, a cuyos efectos será presentado en la Universidad Autónoma de Madrid. El trabajo ha sido realizado bajo su dirección, autorizando su presentación ante el Tribunal Calificador.

Y para que así conste se extiende el presente certificado,

Madrid, diciembre 2009.

V° B° de la Co-Directora y Tutora:

Mercedes Robledo Batanero

Melchor Fernández Almagro, 3
E-28029 Madrid
Tel. +34 91 224 69 00
Fax +34 91 732 80 80
www.cnio.es

cnio
Centro Nacional
de Investigaciones
Oncológicas

Dra. Cristina Rodriguez González de Antona, Investigadora del Grupo de Cáncer Endocrino Hereditario del Centro Nacional de Investigaciones Oncológicas (CNIO), como Directora

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V° B° de la Directora de la Tesis:

Cristina Rodríguez González de Antona

Melchor Fernández Almagro, 3
E-28029 Madrid
Tel. +34 91 224 69 00
Fax +34 91 732 80 80
www.cnio.es

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INDEX

ABBREVIATIONS

5S rRNA	5S ribosomic RNA
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1
ADRs	Adverse drug reactions
BPH	Benign prostatic hyperplasia
BSA	Body surface area
CHOP	cyclophosphamide, hydroxydaunorubicin (Adriamycin), Oncovin (vincristine), and prednisone/prednisolone
CNV	Copy number variation
CR	Complete response
CYP2C8	Cytochrome P450, family 2, subfamily C, polypeptide 8
CYP3A	Cytochrome P450, family 3, subfamily A
EMT	Epithelial to mesenchymal transition
FIGO	International Federation for Gynecology and Obstetrics
GST	Glutathione S-tranferases
GUS	b-glucuronidase
GWAS	Genome wide association studies
HIF-1	Hypoxia induced factor I
HR	Hazard ratio
MDRI	Multidrug resistance protein (ABCB1)
miRNA	microRNA
miR-200	microRNA-200 family
mRNA	messenger RNA
OATP1B1	organic anion transporting polypeptide 1
OATP1B3	organic anion transporting polypeptide 3
OR	Odds ratio
PD	Pharmacodynamics
PD	Progressive disease
P-gp	P-glycoprotein (ABCB1, MDR1)
PK	Pharmacokinetics
PR	Partial response
qRT-PCR	Quantitative real time PCR
SD	Stable disease
SLCO1B1	solute carrier organic anion transporter family, member 1B1 (OATP1B1)
SLCO1B3	solute carrier organic anion transporter family, member 1B3 (OATP1B3)
SD	Standard deviation
SNP	Single nucleotide polymorphisms
ST	Sulphotransferases
T2	T-stage 2
TPMT	Thiopurine S-methyltransferase
UGT	Uridine diphosphate glucuronosyltransferases
UTRs	Untranslated region
WB	Western blot
WT	Wild-type
ZEB1	zinc finger E-box binding homeobox protein 1
ZEB2	zinc finger E-box binding homeobox protein 2

ABSTRACT/RESUMEN

Taxanes (paclitaxel and docetaxel) are used in the treatment of a variety of solid tumours, such as breast, lung, prostate and ovarian cancer. There is a large interindividual variation in the efficacy and adverse effects of these drugs, which might be explained, in part, by the extensive variability in taxanes clearance and/ or by alterations in the therapeutic target. However, at the moment, there are no molecular markers able to predict the major side-effects of these drugs and their efficacy. The polymorphic cytochromes P450 (CYP) are the key enzymes catalyzing taxanes metabolism (CY2C8 and CYP3As for paclitaxel, and CYP3As for docetaxel) and the therapeutic effect of taxanes is meditated through their binding to β-tubulin.

In addition to docetaxel hydroxylation, CYP3As also catalyze the hydroxylation of androgens to less active metabolites. Thus, *CYP3A* polymorphisms might modify the efficacy of docetaxel in prostate cancer (PC) treatment by its inactivation in the tumoral cells and it could influence PC risk through alteration of androgen metabolism. We established the expression of CYP3A4, CYP3A5, CYP3A7, and CYP3A43 in non-tumoral and tumoral prostate samples. We found that only *CYP3A5* was expressed at relevant levels and that its expression was influenced by *CYP3A5*3* polymorphism, while tumoral prostate tissue lacked CYP3A5. Our data suggests that CYP3A5 might be important in the regulation of prostate cell growth by modulation of intra-prostatic androgen levels and that *CYP3A5* polymorphisms could be the functional genetic variations associated with PC. However, it is unlikely that *CYP3A5* polymorphisms could directly influence docetaxel efficacy in PC.

Taxanes target β-tubulin and alterations in the expression patterns of β-tubulin isoforms have been associated with taxanes resistance, especially the over-expression of class III β-tubulin. The regulation of the different β-tubulin isotypes remains largely unknown. However, microRNAs, particularly the miR-200 family, have been suggested to *in vitro* modify taxanes sensitivity through alteration of β-tubulin III expression. We quantified the expression of the miR-200 family and the protein content of β-tubulin isotypes I, II and III in 72 samples of ovarian carcinoma from patients treated with paclitaxel-carboplatin. We found that miR-200 family determined the final β-tubulin III expression and that it was associated with response to treatment and progression-free survival. Thus, this family of microRNAs could constitute a biomarker of response to treatment for ovarian cancer patients.

Neurotoxicity is the most relevant dose-limiting toxicity of paclitaxel. It exhibits substantial interindividual variability of unknown molecular basis which, at the moment, cannot be predicted. Thus, we aimed to identify polymorphisms associated with an increased risk of paclitaxel neurotoxicity. For this purpose we selected 13 relevant polymorphisms in genes encoding paclitaxel metabolizing enzymes and transporters

and genotyped them in 118 Spanish cancer patients treated with paclitaxel. We found an association for *CYP2C8* and *CYP3A5* polymorphisms with paclitaxel neurotoxicity. These genetic variants might be used to inform treatment selection, providing the basis for an individualized paclitaxel pharmacotherapy.

Los taxanos (paclitaxel y docetaxel) se usan en el tratamiento de varios tipos de tumores sólidos (mama, pulmón, próstata y ovario). Existen grandes diferencias interindividuales tanto en la eficacia como en los efectos adversos de estos fármacos que, en parte, podrían ser explicados por la gran variabilidad que existe en la eliminación de los taxanos y/o por alteraciones en su diana terapéutica. Sin embargo, en estos momentos no existen marcadores moleculares capaces de predecir los efectos secundarios de los taxanos y su eficacia. Los citocromos P450 (CYP) son los enzimas clave que catalizan el metabolismo de los taxanos (CYP2C8 y CYP3A para el paclitaxel, y CYP3A para el docetaxel) mientras que el efecto terapéutico está mediado por su unión a la β-tubulina.

Además de hidroxilar el docetaxel, los CYP3A catalizan la hidroxilación de los andrógenos a metabolitos menos activos. Por tanto, los polimorfismos *CYP3A* podrían modificar la eficacia del docetaxel en cáncer de próstata (CP) mediante su inactivación en las células tumorales y podrían influir en el riesgo de CP alterando el metabolismo de los andrógenos. Para investigar esta hipótesis determinamos la expresión del CYP3A4, CYP3A5, CY3A7 y CYP3A43 en muestras de próstatas no-tumorales y tumorales. Descubrimos que sólo el *CYP3A5* se expresaba a niveles relevantes en próstata y que su expresión estaba afectada por el polimorfismo *CYP3A5*3,* mientras que la expresión del CYP3A5 desaparecía en el tejido tumoral. Nuestros datos sugieren que el CYP3A5 podría ser importante para la regulación del crecimiento celular prostático mediante la modulación de los niveles intra-prostaticos de los androgenos y que los polimorfismos en el *CYP3A5* podrían ser las variantes genéticas funcionales asociadas con el CP. Por otra parte, parece improbable que los polimorfismos del *CYP3A5* influyan directamente en la eficacia del docetaxel en el CP.

La diana de los taxanos es la β-tubulina y alteraciones en la expresión de los isotipos de β-tubulina se han asociado con la resistencia a taxanos, especialmente con la sobre-expresión del isotipo III. La regulación de los distintos isotipos es desconocida pero existen evidencias *in vitro* que sugieren que microARNs, fundamentalmente la familia miR-200, modifican la sensibilidad a taxanos alterando la expresión de la β-tubulina III. Cuantificamos la expresión de la familia miR-200 y la expresión proteica de los isotipos I, II y III de la β-tubulina en 72 muestras de pacientes de cáncer de ovario tratados con paclitaxel-carboplatino, y descubrimos que la familia miR-200 determinaba la expresión final de β-tubulina III. Además, estos microARNs se asociaron con la respuesta al tratamiento y la supervivencia libre de recaída de las pacientes. Por lo tanto, esta familia de microARNs podría servir como biomarcador de respuesta al tratamiento en pacientes con cáncer de ovario.

Resumen

La toxicidad limitante de dosis del paclitaxel es la neurotoxicidad, la cual presenta grandes diferencias interindividuales y tiene una base molecular desconocida que la hace impredecible. Por tanto, nos fijamos el objetivo de identificar polimorfismos asociados al riesgo de neurotoxicidad por paclitaxel. Para ello elegimos 13 polimorfismos relevantes en genes que codifican los enzimas de metabolización y transporte del paclitaxel y los genotipamos en 118 pacientes españoles de cáncer tratados con este fármaco. Encontramos una asociación estadísticamente significativa entre polimorfismos del *CYP2C8* y *CYP3A5* y la neurotoxicidad del paclitaxel. Estas variantes genéticas podrían servir para mejorar el tratamiento con paclitaxel, proporcionando la base para una farmacoterapia individualizada.

INTRODUCTION

1. Pharmacogenetics

There are large interindividual differences in the way patients respond to medication. This applies to both drug efficacy and toxicity. Drug inefficacy is a major clinical problem; for many drugs the proportion of patients not responding to standard treatments ranges from 30-70% (Table 1). On the other hand, serious adverse drug reactions (ADRs) contribute to 7% of all hospitalizations and 100,000 deaths per year in USA (Eichelbaum, et al. 2006) and in United Kingdom, ADRs caused 6,5% of hospitalizations in 2002 (Pirmohamed, et al. 2004).

Table 1. Examples of decreased drug efficacy[1]

Disease	Drug Class	Non responders (%)
Asthma	β2adrenergic agonist, 5-LO, LTD4	4 - 75
Cancer	Various (breast, lung, brain)	30 - 100
Depression	SSRIs, Tricyclics, MAOs	20 - 40
Diabetes	Sulfonylurea, Biguanides, Glitazones	50 - 75
Duodenal ulcer	H2antagonists, Proton pump inhibitors	20 - 70
Hyperlipidemia	HMGCoA reductase, Resins, Niacin	30 - 75

[1](Kalow 2001)

As it is shown in the simplified diagram shown in figure 1, using standard drug treatments patients can be divided into four major groups according to the drug effects: responders without toxicity, non-responders without toxicity, responders with toxicity and non-responders with toxicity. A prospective identification of patients most likely to benefit from specific therapies would greatly improve drug therapy. However, this identification is still not possible for many drugs (McLeod and Evans 2001). Part of this variability in drug outcome can be explained by non-genetic factors, such as age, sex, concomitant illnesses, drug interactions etc. However, nowadays we know that inherited genetic differences can also have a great influence in both efficacy and toxicity of drug treatments (Evans and McLeod 2003; Evans and Relling 1999). These genetic variants include single nucleotide polymorphisms (SNPs), nucleotide deletions, insertions, and gene copy number variations (CNVs) that could affect gene expression or function (McLeod and Evans 2001). Examples of genetic variation associated with drug outcome include: Cytochrome P450 2D6 (CYP2D6) for antidepressants and antipsychotics (Gurwitz and Weizman 2004), CYP2D6 for the oncology drug tamoxifen

(Dawood and Leyland-Jones 2009) and CYP2C9 for the vascular disease drug warfarin (Evans and Relling 2004).

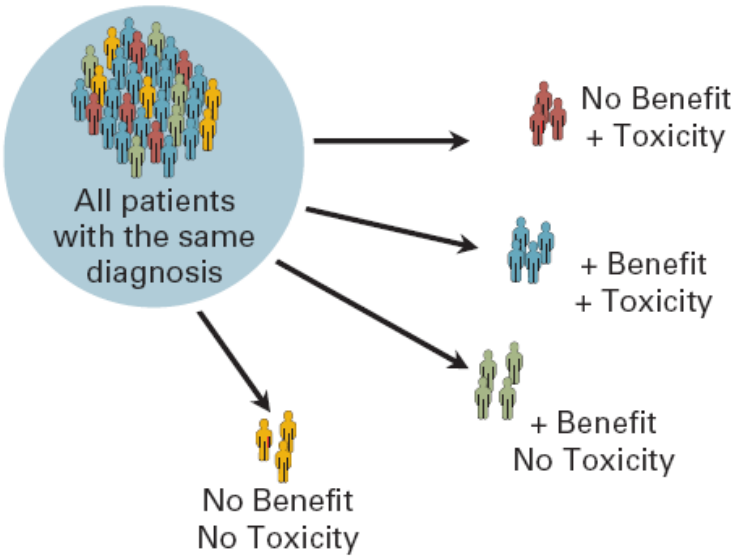

Figure 1. Variability of response to standard drug treatments. Patients could be divided into four major groups according to their response to the drug: responders without toxicity, non-responders without toxicity, responders with toxicity and non-responders with toxicity.

Thus, lack of drug efficacy and drug toxicity are among the major problems of modern medicine, and efforts to discover biomarkers predictive of drug outcome are critical for therapy improvement. The aim of pharmacogenetics is to individualize pharmacotherapy by identifying genetic variations that modify therapeutic efficacy and/or adverse effects of drugs. In this way drug doses could be adjusted and/or alternative therapies applied according to a genetic variation, personalizing drug therapy (Dawood and Leyland-Jones 2009; Evans and Relling 2004).

There are already several clinically accepted pharmacogenomic biomarkers, such as Thiopurine S-methyltransferase (TPMT) genetic variations for 6-mercaptopurins myelotoxicity and HLA-B*5701 for Acabavir Stevens-Johnson syndrome, which serve as predictors of drug outcome (Ingelman-Sundberg 2008). Unfortunately, nowadays one-size-for-all approaches are still used for most drug treatments, even for drugs with narrow therapeutic indexes.

1.1. Cancer pharmacogenetics

In oncology the therapeutic window, separating drug toxicity from optimal treatment, is often narrow. The reason is that, although ideally, anticancer drugs should

be active exclusively on cancer cells and not cause toxicity in normal tissues, in general, this is not the case and anticancer drugs are usually aggressive. In addition, lack of efficacy can have critical consequences for the patients. Thus, pharmacogenetics is especially relevant in Oncology, were lack of efficacy and dose-limiting toxicities are among the major obstacles to improve the survival of cancer patients (Dawood and Leyland-Jones 2009; Ingelman-Sundberg 2008).

1.2. Germinal and somatic genetic variants and their relevance for pharmacokinetics and pharmacodynamics

Pharmacokinetics (PK) and Pharmacodynamics (PD) can be considered as a range of continuous events starting by drug ingestion and ending with the clinical effect, with variability in the pharmacokinetic processes modifying the pharmacodynamic result. PK describes the association between time and the concentration of the drug and metabolites in plasma, and it is constituted by the processes of drug absorption, distribution, metabolism, and excretion (Dawood and Leyland-Jones 2009). The most relevant genes for PK are those coding the Cytochrome P450 (CYP) enzymes in families 1-3. It has been estimated that CYP3A4, CYP2D6 and CYP2C9 participate in the metabolism of 50, 25 and 15%, respectively, of drugs in commercial use. CYP3A4 is the most abundant hepatic CYP and it has a broad substrate specificity. The CYP enzymes participate in the phase I metabolism of drugs (Evans and Relling 1999; Shimada, et al. 1994; Wrighton and Stevens 1992) while for phase II metabolism, involving primarily conjugation, the most relevant enzymes are the uridine diphosphate glucuronosyltransferases (UGT), sulphotransferases (ST) and glutathione S-tranferases (GST) (Burchell 2003). Drug transporters mediate drug uptake and efflux, the most important genes for uptake include the organic anion-transporters (OAT) (Gui, et al. 2008; Smith, et al. 2005), the organic anion-transporting polypeptides (OATP) and organic cation transporters (OCT) and for efflux P-glycoprotein (P-gp) together with other multiple drug resistance proteins (MRD) (Chan, et al. 2004). PD comprises the effect of the drug, implying drug response and toxicity (Dawood and Leyland-Jones 2009). The most relevant genes for PD are the therapeutic drug targets, but can also include MDR1 (coding P-gp), p53, apoptosis genes etc (Dawood and Leyland-Jones 2009). An alteration in the PKs and/or PDs of a drug can lead to toxicity and/or poor drug response (Dawood 2009).

Heritable genetic factors (germinal variation) and tumor genomic factors (somatic variation) can both lead to interindividual differences in drug outcome. PK is mainly affected by germline polymorphisms, while PD can be frequently influenced by both germline and somatic variants (Figure 2).

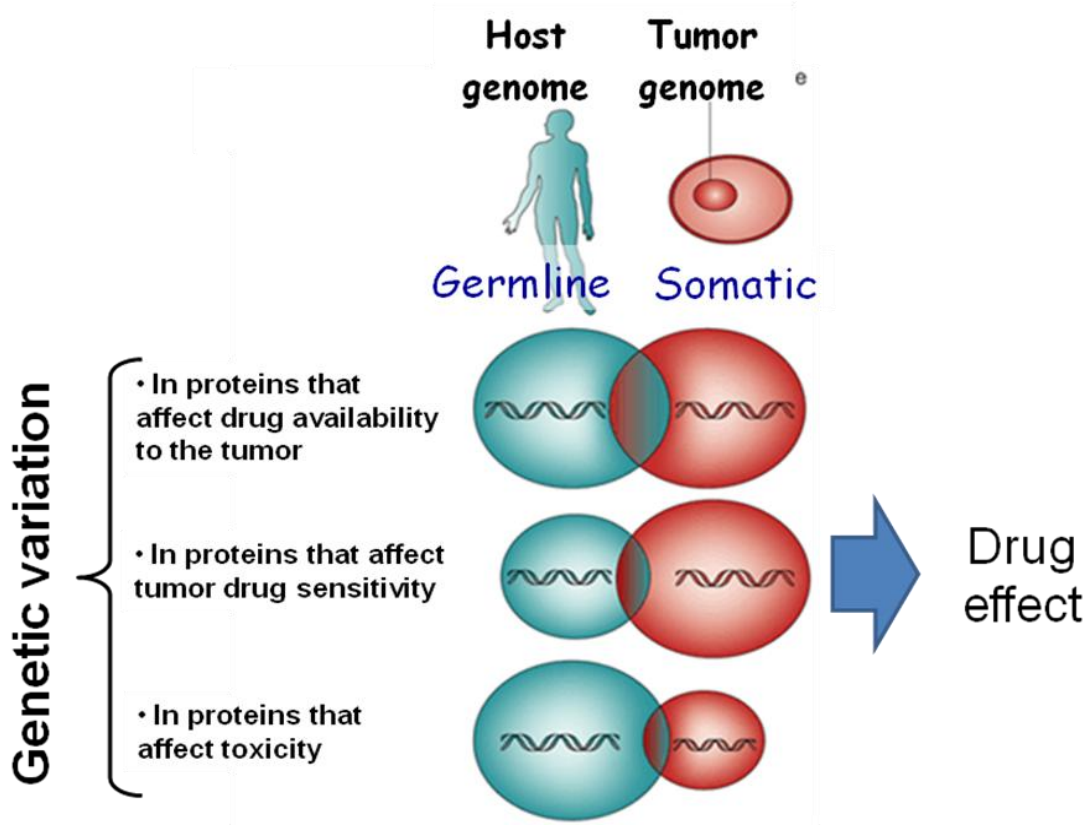

Figure 2. Germline and somatic variation can both influence drug effects.

1.3. Candidate gene studies vs. genome wide association studies (GWAS)

Most of the pharmacogenetic studies so far performed have been based on candidate gene approaches, focused on pathways and on polymorphisms in relevant genes with a biological relationship with the drug of interest. During recent years, our knowledge on drug PK and PD pathways has increased immensely and for many drugs we know now the implicated metabolizing enzymes, transporters and therapeutic targets as well as the metabolites and their therapeutic activities. Carefully planned candidate gene studies focused on complete pathways, instead of using just a few key players, is an important tool in Pharmacogenetic studies (Evans and Relling 2004). Thus, this strategy can uncover the underlying genetic causes for differences on treatment outcome for many drugs.

However, candidate gene studies are limited by current knowledge, while for the recently developed genome wide association studies (GWAS), no prior biological knowledge is required and all the genome is analyzed. This approach can thus further clarify metabolic routes, transporter functions and secondary targets that remain still unknown (Nelson, et al. 2009). Therefore, the combination of these strategies will be the best way to proceed in the pharmacogenetics field in the future.

2. Taxanes

2.1. Clinical use and mechanism of action

Taxanes are administered to more than ¼ of the patients treated with anticancer drugs in USA. They are frequently used as first line chemotherapy in the treatment of breast, ovary, lung and prostate cancer usually in combination with other drugs. The two commonly used taxanes are docetaxel (taxotere) and paclitaxel (taxol) (Figure 3).

Paclitaxel, an extract from the bark of the Pacific yew, *Taxus brevifolia*, is a chemotherapeutic drug widely used in the treatment of a variety of solid tumors, such as breast, ovarian and lung cancer (Rowinsky 1997). Docetaxel is a semi-synthetic derivative from extracts of the needles of the European yew tree (*Taxus baccata*), which has greater affinity for β-tubulin, and a different microtubule polymerization pattern (Diaz and Andreu 1993; Saloustros, et al. 2008). Docetaxel is mainly used in the treatment of breast, lung, and metastatic prostate cancers (De Ligio, et al. 2009; Petrylak, et al. 2004).

Taxanes stabilize cellular microtubules through β-tubulin binding, which alters cell motility, transport and cell division, and consequently leads to cell death (Schiff, et al. 1979; Yvon, et al. 1999).

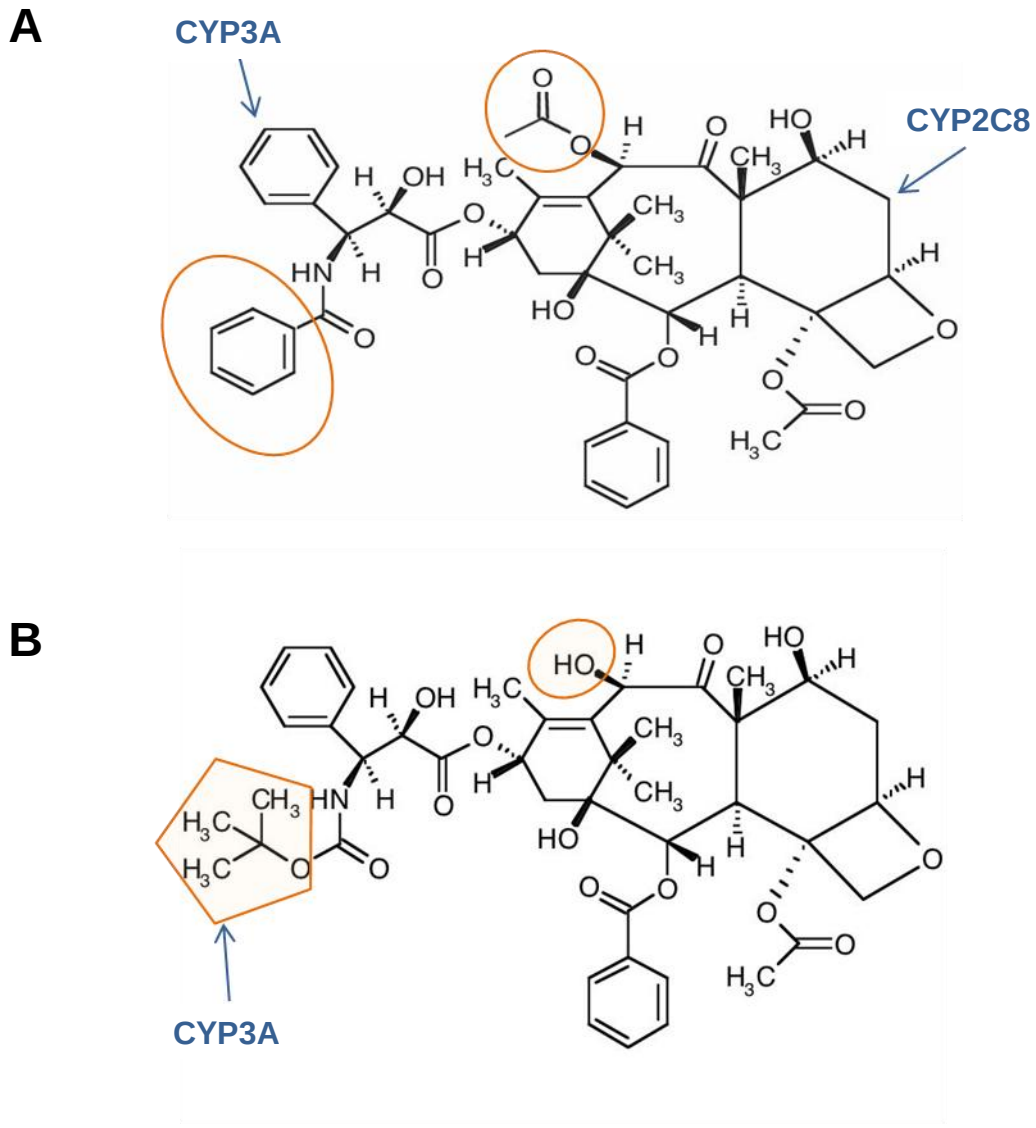

Figure 3. Chemical structure of taxanes A) paclitaxel and B) docetaxel. The differences among the molecules are highlighted. The positions for the hydroxylation and the enzymes involved are shown.

2.2. Clinical problems of taxanes

2.2.1. Toxicities

The major side-effects of paclitaxel are hematologic toxicity (mainly neutropenia) and peripheral neuropathy, while for docetaxel the dose limiting toxicity is hematologic. The risk of neutropenia has been significantly reduced in recent years by using shorter infusion times and by the administration of granulocyte colony stimulating factors (Eisenhauer, et al. 1994; Rowinsky, et al. 1993a). On the other hand, paclitaxel

neurotoxicity cannot be prevented nor treated, it can last for months, and in severe cases it may cause irreversible nerve damage (Chaudhry, et al. 1994; Lipton, et al. 1989). Neurotoxicity, which has become the dose-limiting toxicity of paclitaxel, exhibits substantial interindividual variability. This toxicity is dose-dependent and more frequent in weekly paclitaxel regimens (Argyriou, et al. 2008; Seidman, et al. 2008), in patients with diabetes, prior neurotoxic chemotherapy treatments and in patients with pre-existing neuropathies (Mielke, et al. 2005; Rowinsky, et al. 1993b). Age and gender might also be risk factors (Akerley, et al. 2003; Mielke, et al. 2003; Mielke et al. 2005) and also decreased levels of plasma nerve factors might play a role in the neurotoxicity (Cavaletti, et al. 2004). However, a large part of the variability in the neurotoxicity exhibited by patients remains unexplained and is unpredictable.

Interestingly, an association between paclitaxel neurotoxicity and PK parameters has been demonstrated (Green, et al. 2009; Mielke et al. 2005), suggesting an important role for drug metabolism and transport in this adverse effect (Figure 3). In addition, the influence of polymorphisms in paclitaxel PK-related genes on the drug neurotoxicity has been investigated in three previous studies using different strategies and producing contradictory results. One study found higher neurotoxicity for a variant allele of *CYP2C8,* (Green et al. 2009) while Sissung *et al.* (Sissung, et al. 2006) and Marsh *et al.* (Marsh, et al. 2007) did not find significant associations for the genes studied. However, two studies had less than 25 patients recruited, and the latter did not take into account the dose-dependency nature of paclitaxel neurotoxicity. This indicates that, despite the important implications on clinical practice, the impact of genetic variation on paclitaxel neurotoxicity is still unclear.

2.2.2. Efficacy

Despite all the improvements that the use of taxanes has brought to cancer treatment, drug resistance, both primary and acquired, is still a major problem for many patients and it is exhibited as lack of response or relapse. Resistance to taxanes has been reported in various tumour types: ovarian (Heintz, et al. 2006; Ozols, et al. 2003), breast (Paradiso, et al. 2005) and lung (Seve and Dumontet 2005). Different mechanisms have been suggested to cause the resistance, such as chromosomal instability (Swanton, et al. 2009), overexpression of P-gp (Horwitz, et al. 1993), over-expression of class III β-tubulin (Mozzetti, et al. 2005) and mutations in the target of the drug, the β-tubulins (Giannakakou, et al. 1997), however, recent studies have

discarded this latter possibility (Sale, et al. 2002). Thus, the identification of resistance factors and critical determinants of antitumor efficacy of microtubule-stabilizing agents is essential to improve the therapeutic efficacy of taxanes.

3. Taxane metabolism and transport

Taxanes are administered by intravenous injection, and their elimination is mediated by hepatic metabolism and biliary excretion (Figure 4). A great interindividual variability on taxanes PK has been reported both for paclitaxel (Henningsson, et al. 2005; Smith, et al. 2007; Somlo, et al. 2001) and for docetaxel (Baker, et al. 2009; Bosch, et al. 2006; Tran, et al. 2006). This variability has unknown molecular basis, but there are evidences suggesting that genetic variation affecting taxanes metabolism and/or transport could underlie these differences (Green et al. 2009; Rodriguez-Antona, et al. 2008; Smith et al. 2007).

3.1 Hepatic metabolism

In the liver paclitaxel is hydroxylated at the 6α position by CYP2C8, forming the major metabolite, and at the C3´ position by CYP3A4/5 (Rahman, et al. 1994; Vaclavikova, et al. 2004) (figure 4). For docetaxel, CYP3A4/5 catalyze the hydroxylation in the ter-butyl side chain, subsequently, and in a minor manner, an unstable aldehyde can be formed that is converted to oxazolodinones (Vaclavikova et al. 2004)

These CYP metabolizing enzymes are polymorphic. All the so far described genetic variants in these genes are summarized in http://www.cypalleles.ki.se/. For *CYP2C8* there are several SNPs affecting functionality: *CYP2C8*3* (R139K; K399R) exhibits an altered activity (Aquilante, et al. 2008; Dai, et al. 2001; Kirchheiner, et al. 2008; Niemi, et al. 2005; Niemi, et al. 2003; Soyama, et al. 2001) and haplotypes B and C, represented by *CYP2C8*1B* (rs7909236) and rs1113129 confer an increased and reduced activity, respectively (Rodriguez-Antona et al. 2008).

For *CYP3A4* there are no common coding polymorphisms and the only SNP associated with altered activity in part of the studies is the promoter *CYP3A4*1B* allele (Rodriguez-Antona, et al. 2005). On the other hand, *CYP3A5* is very polymorphic. Its

activity in Caucasians is determined by *CYP3A5*3* allele through alternative splicing (Kuehl, et al. 2001), while other defective alleles, *CYP3A5*6* and *CYP3A5*7*, are present only in Africans (Hustert, et al. 2001; Kuehl et al. 2001; Lee, et al. 2003; Lin, et al. 2002).

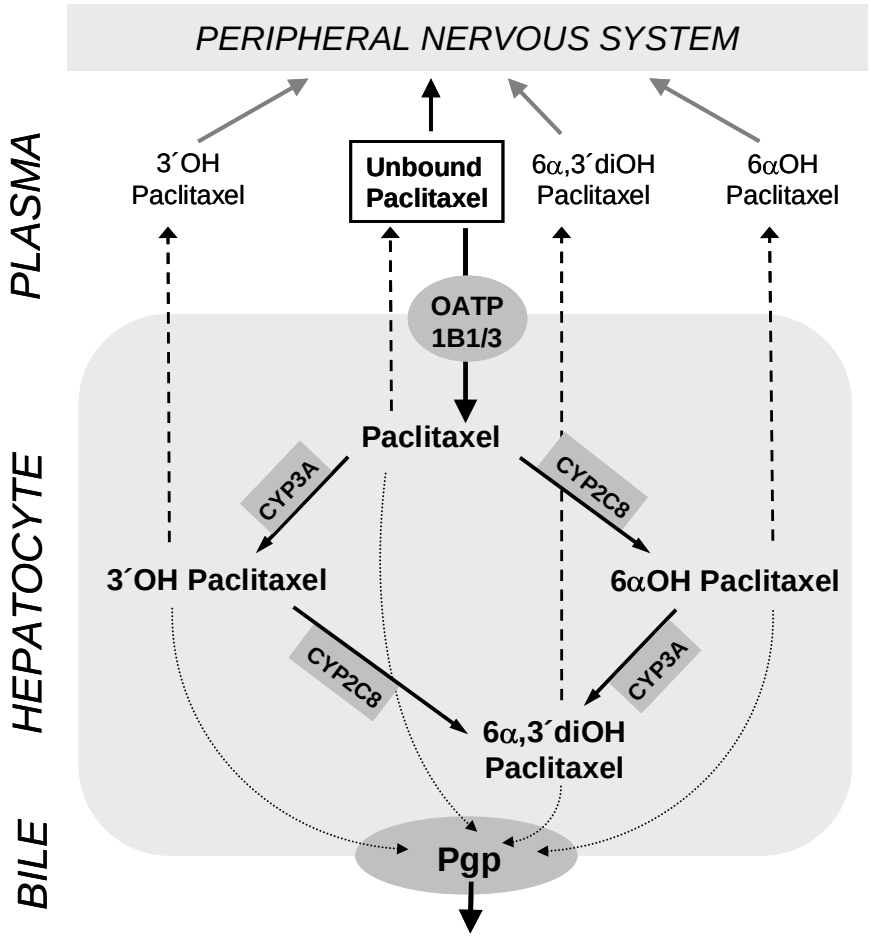

Figure 4. Paclitaxel hepatic metabolism. Paclitaxel enters the hepatocyte via OATP1B1 and/or OATP1B3. In the hepatocyte, it is metabolized by CYP2C8 to 6α-hydroxypaclitaxel, which is the major metabolite, and by CYP3A to metabolite C3´-hydroxypaclitaxel. Paclitaxel and its metabolites either diffuse to plasma or are excreted to the biliar canaliculi by P-glycoprotein.

3.2 Metabolism in tumoral tissues

The presence in tumoral tissues of CYPs relevant for taxanes metabolism has been investigated in some studies. These enzymes could not affect the PK of the drug, but might be important for the efficacy by altering the drug metabolism inside the cancer cells (e.g. by metabolizing the drug to less active molecules). For example, an

association between low expression of CYP3A4 in breast tumors and a better response to docetaxel has been reported (Miyoshi, et al. 2002), a high tumoral expression of CYP3A4 in peripheral T-cell lymphomas has been significantly associated with a lower complete remission of patients treated with the standard multiagent chemotherapy CHOP (Rodriguez-Antona, et al. 2007) and a high CYP3A expression in osteosarcoma tumours has been associated with metastasis and poor prognosis, although only 18 patients were available to perform this analysis (Dhaini, et al. 2003).

In some studies CYP3A4 or CYP3A5 expression has been detected in tumoral tissues, for example in prostate (Moilanen, et al. 2007; Murray, et al. 1995b; Zhang, et al. 2006), breast (Kapucuoglu, et al. 2003; Modugno, et al. 2003) and ovarian cancer samples (DeLoia, et al. 2008; Downie, et al. 2005). CYP2C8 expression has been reported in breast (Knupfer, et al. 2004) and ovarian cancers (DeLoia et al. 2008). Then again, lack of expression or very low levels of these genes have been detected in cancer samples in other studies: CYP3A4 in prostate (Westlind, et al. 2001) and ovarian cancers (DeLoia et al. 2008), CYP3A4/5 in breast (Modugno et al. 2003; Oyama, et al. 2005) and no CYP2C8 was detected in studies on breast cancer (Modugno et al. 2003). Thus, due to these controversial results, further studies are needed to identify the relevance of CYP2C8 and CYP3A enzyme(s) expression in tumoral tissues, with more homogenous tumour types as well as techniques able to accurately measure the expression at different levels (most of these studies were performed exclusively at mRNA level).

3.3. Hepatic transport

Taxanes uptake into the hepatocytes is mediated by the organic anion transporting polypeptide (OATP) 1B3 and, to some extent, OATP1B1 (Gui et al. 2008; Smith et al. 2005) and efflux is mediated by P-gp (Figure 4) (Sparreboom, et al. 1997; Walle and Walle 1998) and also by MRP2 for docetaxel (Huisman, et al. 2005). The genes encoding OATP1B1/3 and P-gp are subjected to relevant genetic variation, which can alter drug metabolism and disposition, as it has been shown previously (Leschziner, et al. 2007; Rodriguez-Antona et al. 2008; Rodriguez-Antona et al. 2005; Smith et al. 2007). For the uptake transporters OATP1B3 and OATP1B1 three missense polymorphisms with reported functional consequences are known (Smith et al. 2007), while for *ABCB1* mainly three variants (1236C>T, 2677G>T, 3435C>T) (Leschziner et al. 2007) have been associated with altered transport.

3.4. Transport in tumoral cells

Both docetaxel and paclitaxel are substrates of P-gp together with other anti-cancer drugs such as vinca alkaloids, anthracyclines and imatinib (Huang 2006). Over-expression of P-gp (encoded by *ABCB1* multidrug resistance gene) in tumor cells has been associated with resistance to taxanes *in vitro* (Horwitz, et al. 1986; Takano, et al. 2009), although demonstrating its role *in vivo* as a clinical marker for drug response has been problematic and remains questioned (Chen, et al. 2009; Chevillard, et al. 1996; Fojo and Menefee 2007). Thus, its relevance in patients remains unclear (Seve and Dumontet 2008). Another efflux transporters, MRP7 (Hopper-Borge, et al. 2004; Shen, et al. 2009) and MRP2 (Huisman 2005) have also been related to resistance to taxanes *in vitro* but their role *in vivo* has not been determined.

Therefore, alterations in taxanes metabolism and transport could have a critical importance for their toxicity and/or efficacy. Both germline and somatic variations altering the function of the critical proteins mediating taxanes PK could be markers of taxanes outcome, however, at the moment, their relevance is mainly unknown.

4. Therapeutic target of taxanes

Taxanes are anti-mitotic drugs able to alter the microtubule dynamics by binding to the β-subunits of microtubules. Microtubules are composed of heterodimers of α- and β-tubulins (Figure 5) and are essential for diverse cellular functions, such as cell division, maintenance of cell shape and intracellular trafficking. The binding of paclitaxel or docetaxel to the β-tubulin subunit leads to the stabilization of the cellular microtubules, mitotic arrest and finally to cell death (Seve and Dumontet 2008).

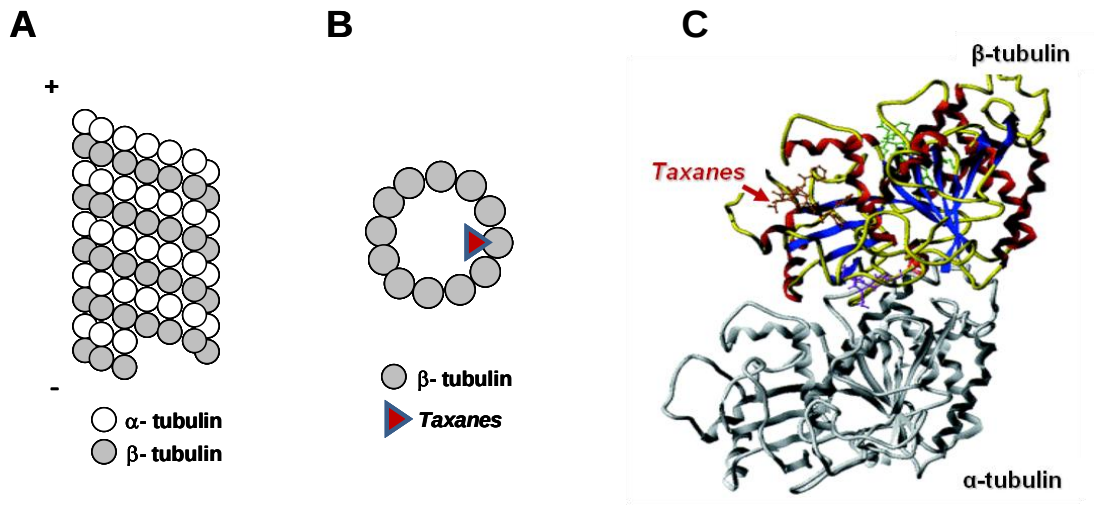

Figure 5. Taxanes target β-tubulins. A) Microtubules are composed of heterodimers of α- and β-tubulin, which are continuously incorporated and released. **B) and C)** Taxanes bind to β-tubulin in the inner surface of the microtubule structure.

4.1. β-tubulin isotypes and their expression in normal and tumoral tissues

There are at least 8 different β-tubulin isotypes expressed in humans (Leandro-Garcia LJ, submitted for publication). These genes are constituted by 4 exons and 3 introns, while the non-functional 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).

Isotypes I and IVb are ubiquitous, but others are tissue-specific and the contribution of the different isotypes to the total β-tubulin content varies in each tissue. The expression pattern differs also between normal and tumoral tissue (Leandro-Garcia LJ). These changes in the expression pattern are believed to play a part in the resistance to microtubule targeting drugs.

The most frequent change is the tumoral overexpression of class III β-tubulin. In normal tissue isotype III is present only in nervous tissue, while its expression has been observed in several tumoral tissues such as breast, ovarian and lung. High tumoral β-tubulin III expression has been associated with worse survival in breast (Seve and Dumontet 2008) head and neck (Koh, et al. 2009), and non small cell lung cancer (Rosell, et al. 2003; Seve, et al. 2005b), which may indicate that β-tubulin III expression

could be used as a prognostic predictor marker. In addition, class III tumoral over-expression has been demonstrated *in vitro* to be related to taxanes resistance (Hari, et al. 2003; Kavallaris, et al. 1999). In fact, β-tubulin III has also been associated with resistance to taxanes in patients (Seve and Dumontet 2008). All together, β-tubulin III could act as a prognostic and a treatment predictive factor, depending on the tumor type and other clinical variables.

Specifically, for ovarian carcinoma, which is on the main topics of this Thesis, high levels of classes I and IV, intermediate levels of class III and low levels of class II β-tubulin protein expression have been reported by Ohishi *et al.* (Ohishi, et al. 2007). An increased expression of β-tubulin III in ovarian carcinoma has also been associated with worse overall survival (Ferrandina, et al. 2006) and response to treatment (Kavallaris, et al. 1997; Mozzetti et al. 2005; Umezu, et al. 2008), although Aoki *et al.* reported a better survival for patients on taxane-based regimens with ovarian clear cell adenocarcinoma expressing class III (Aoki, et al. 2009). On the other hand, absence of β-tubulin II expression has been associated with advanced stage and short progression free survival (Ohishi et al. 2007).

4.2. Regulation of β-tubulin isotypes

The regulation of the expression of the different β-tubulin isotypes remains largely unknown. The molecular mechanisms leading to the up-regulation of class III β-tubulin in tumors are becoming the subject of increasing number of investigations. Hypoxia induced factor I (HIF-1) seems to play a role in the regulation of class III β-tubulin in ovarian carcinomas (Raspaglio, et al. 2008), and epigenetic modifications have also been suggested to be involved in its over-expression, in ovarian tumors (Izutsu, et al. 2008) and in melanoma cells (Akasaka, et al. 2009). A recent study by Cochrane *et al.* suggested an important role for microRNAs (Cochrane, et al. 2009).

4.2.1. MicroRNAs regulating β-tubulin expression

MicroRNAs (miRNAs) are small, 20–22 nucleotide long, non-coding RNAs that usually act as endogenous repressors of gene activity. They bind to partially complementary sites, usually in the 3′ UTRs of mRNAs, producing inhibition of translation and some level of degradation of the target mRNA. Over 850 mature human miRNA sequences are represented in the Sanger database version 13.0 and they are

predicted to regulate expression of at least 30% of genes in humans. miRNAs have roles in important cellular processes including development, differentiation, proliferation and apoptosis. They can be involved in the initiation and progression of cancer and both losses and gains of miRNA function have been shown to contribute to cancer development, thus, they can function as both, tumor suppressors and oncogenes. miRNA expression can be deregulated in cancer by a variety of mechanisms including amplification, deletion, mutation, and epigenetic silencing (Croce 2009; Cho 2007).

Cochrane *et al.* have shown *in vitro* that increased levels of miR-200c cause a decrease in class III β-tubulin expression (Cochrane et al. 2009). miR-200c is part of the miR-200 family which is formed by five miRNAs located in two clusters in chromosomes 1 and 12 in the human genome (Gregory, et al. 2008). The 200b/c/429 miRNAs are predicted to share the same target sites based on the similarity of their seed sequences, while 1 nucleotide change in the seed sequence of miR-141 and miR-200a mark some differences in their targets (Gregory et al. 2008). The miR-200c family is involved in the epithelial to mesenchymal transition (EMT) and they have been shown to regulate E-cadherin expression via suppression of zinc finger E-box binding homeobox proteins 1 and 2 (ZEB1 and ZEB2) (Gregory et al. 2008; Korpal, et al. 2008; Park, et al. 2008). High expression of miR-200c restores *in vitro* the sensitivity to microtubule targeting agents (Cochrane et al. 2009). In a recent study, a high expression of miR-200a, miR-200b and miR-429, all in the same cluster, was associated with improved ovarian cancer survival (Hu, et al. 2009), however, in another study high expression of the miRNAs from the miR-200 family correlated with poor survival (Nam, et al. 2008). Thus, further studies are needed to clarify the importance of microRNAs for tubulin expression and to explore their association with taxanes treatment outcome.

In conclusion, many remarkable discoveries have been made in the recent years on the pharmacogenetics field: using different approaches various genetic variants have been associated with relevant drug-related toxicities and microRNAs have been shown to play a key role not only in the tumor biology but also in drug therapeutic responses. As for taxanes, at the present time, there are no clinical markers of therapy outcome and it is impossible to predict neither efficacy nor toxicity. Variations affecting taxanes PK and /or PD, either through genetic variations or alteration in the regulation of key genes, could play a critical role in therapy outcome. Previous investigations have attempted to approach this problem, but studies

conducted with non-functional SNPs, lack of characterization of full haplotypes, heterogenous patient populations and sometimes lack of essential gene expression data in the target tissue, have hindered these studies. Thus, there is a need to identify biomarkers of taxanes outcome. These could help to individualize therapies and would have a great social impact, improving and prolonging the life of a significant number of cancer patients.

INTRODUCCIÓN

1. *La farmacogenética*

Las bases moleculares que determinan la toxicidad de un medicamento, o cualquier otro xenobiótico, tienen una gran relevancia clínica, ya que se ha estimado que las reacciones adversas a fármacos constituyen entre la cuarta y sexta causa de muerte más frecuente en los países desarrollados. Asimismo, la falta del efecto terapéutico hace su uso inútil. En este contexto, el fondo genético de cada individuo puede jugar un papel decisivo en la respuesta a un fármaco. Esta es la base de la Farmacogenética, que estudia las variaciones genéticas que modifican la eficacia terapéutica y/o toxicidad de un fármaco, ayudando a seleccionar el tratamiento farmacológico más seguro y eficaz para cada paciente (Eichelbaum et al. 2006; Evans and Relling 2004; Pirmohamed et al. 2004). Las variaciones genéticas incluyen polimorfismos puntuales de un nucleótido (SNP), pequeñas inserciones, deleciones o variaciones en el número de copia de los genes (McLeod and Evans 2001) y pueden ser germinales o somáticas. A pesar de los avances en la medicina molecular y el diseño de fármacos dirigidos a dianas celulares específicas, la eficacia clínica de la terapia anticancerosa está limitada de una forma importante por la incapacidad de predecir de una forma precisa la respuesta del tumor y los efectos tóxicos en el paciente a la terapia. Esta incapacidad de predecir los efectos en el paciente tiene una especial relevancia en los tratamientos oncológicos debido a los estrechos índices terapéuticos de estos medicamentos (Dawood and Leyland-Jones 2009; Ingelman-Sundberg 2008).

2. *Taxanos: uso, problemas clínicos y mecanismo de acción*

Los taxanos se utilizan para el tratamiento de varios tipos de tumores sólidos (mama, ovario, próstata, pulmón). Los dos taxanos más comúnmente utilizados son el docetaxel (Diaz and Andreu 1993; Saloustros et al. 2008) y el paclitaxel (Rowinsky 1997). Ambos comparten el mismo mecanismo de acción que consiste en su unión a la β-tubulina y estabilización de los microtúbulos celulares, causando una parada del ciclo celular y entrada en apoptosis (Schiff et al. 1979; Yvon et al. 1999). Los principales problemas clínicos de los taxanos incluyen: la falta de eficacia y las toxicidades neurológicas (paclitaxel) y hematológicas (paclitaxel y docetaxel). Mientras que los problemas asociados a la toxicidad hematológica han disminuido gracias al uso de factores de crecimiento hematopoyéticos (Eisenhauer et al. 1994; Rowinsky et al. 1993a), la neurotoxicidad sigue siendo inmanejable y es el efecto adverso más relevante del paclitaxel (Chaudhry et al. 1994; Lipton et al. 1989). Esta neurotoxicidad, de base molecular desconocida, presenta grandes diferencias interindividuales. Por otra parte, la resistencia a taxanos se ha observado en varios tipos de tumores: ovario

(Heintz et al. 2006; Ozols et al. 2003), mama (Paradiso et al. 2005) y pulmón (Seve and Dumontet 2005). Como causa de la resistencia a taxanos se ha sugerido entre otros mecanismos, la inestabilidad cromosómica (Swanton et al. 2009), la sobre-expresión de la P-glicoproteína y del isotipo III de la β-tubulina (Mozzetti et al. 2005) y mutaciones en las β-tubulinas (Giannakakou et al. 1997), si bien este último ha sido descartado en tumores (Sale et al. 2002). La identificación de factores predictivos, tanto de resistencia como de toxicidad a taxanos, es esencial para mejorar la terapia con estos fármacos.

3. *Metabolismo de taxanos*

Los taxanos se suministran de forma intravenosa y su eliminación está mediada por el metabolismo hepático y la excreción biliar. Existe una gran variabilidad interindividual en la farmacocinética del paclitaxel (Henningsson et al. 2005; Smith et al. 2007; Somlo et al. 2001) y docetaxel (Baker et al. 2009; Bosch et al. 2006; Tran et al. 2006). Esta variabilidad tiene una base molecular desconocida, pero diversos estudios sugieren que variaciones genéticas que afecten el metabolismo y transporte de los taxanos podrían ser relevantes en este proceso (Green et al. 2009; Rodriguez-Antona et al. 2008; Smith et al. 2007). El paclitaxel entra en los hepatocitos vía OATP1B1/3, y se excreta al canalículo biliar mediante la P-glicoproteína. En el hígado el paclitaxel es metabolizado por el CYP2C8 y CYP3A4/5 (Rahman et al. 1994; Vaclavikova et al. 2004) y el docetaxel por el CYP3A4/5 (Vaclavikova et al. 2004). Una alteración en la expresión y actividad de estos enzimas y transportadores, tanto en el hígado como en los tejidos tumorales, podría influir en las toxicidades y eficacia de estos fármacos.

4. *β-tubulinas: expresión y regulación*

Las tubulinas forman los microtubulos celulares, que son los principales componentes del citoesqueleto y que permiten, entre otros procesos la división celular. Las β-tubulinas están codificadas por una familia multigénica que produce proteínas ligeramente distintas, que presentan una alta conservación evolutiva y una expresión específica de tejido. Las células tumorales presentan alteraciones en la expresión de los isotipos de β-tubulina que podrían afectar la sensibilidad a los fármacos de unión a microtúbulos (LJ Leandro et al). Específicamente, una sobre-expresión tumoral del isotipo III se ha relacionado con una resistencia a los fármacos de unión a microtúbulos (Seve and Dumontet 2008). La regulación de los distintos isotipos de β-tubulina es desconocida, pero evidencias *in vitro* sugieren que los microRNAs, en especial el miR-200c, regulan la expresión de la β-tubulina III (Cochrane et al. 2009).

OBJECTIVES/OBJETIVOS

The principal purpose of this thesis was to increase our knowledge on the expression and regulation of taxanes metabolizing enzymes (CYPs) and their therapeutic target (β-tubulin), as well as to identify biological markers associated with taxanes efficacy and toxicity. This data could provide the basis for an individualized taxane pharmacotherapy. To achieve this, we placed the following specific aims:

1. To characterize in a comprehensive manner the CYP3A enzyme(s) expression in normal and tumoral prostate tissue to determine whether these enzymes could directly influence the efficacy of docetaxel treatment in prostate cancer patients.

2. To establish the contribution of microRNAs to β-tubulin protein expression and to determine if they could influence the response and the survival of ovarian cancer patients treated with a paclitaxel-based therapy.

3. To identify genetic markers associated with paclitaxel neurotoxicity by using a pharmacokinetics-based candidate gene approach and to integrate them in a single prediction model to assess the individual risk to develop neurotoxicity.

El objetivo principal de esta tesis fue incrementar nuestros conocimientos sobre la expresión y regulación de los enzimas que metabolizan los taxanos (los CYPs) y de su diana terapéutica (la β-tubulina) y utilizar estos conocimientos para identificar marcadores biológicos asociadas con la eficacia y toxicidad de los taxanos. Estos datos podrían proporcionar una base para la individualización de farmacoterapia con estos fármacos. Este objetivo principal se desglosa en los siguientes objetivos específicos:

1. Caracterizar la expresión de los enzimas CYP3A en tejido de próstata normal y tumoral para determinar si estos enzimas podrían influir directamente en la eficacia del docetaxel en el tratamiento de pacientes con cáncer de próstata.

2. Establecer la contribución de los microARNs a la expresión proteica de la β-tubulina y determinar si éstos podrían influir en la respuesta y supervivencia de pacientes con cáncer de ovario tratados con taxanos.

3. Identificar marcadores genéticos asociados con la neurotoxicidad de paclitaxel utilizando una estrategia de genes candidatos basada en la farmacocinética e integrarlos a un único modelo de predicción para estimar el riesgo individual de neurotoxicidad.

MATERIALS & METHODS

1. Patients and clinical data

The cancer patient samples used in this thesis were collected through close collaborations with Spanish Hospitals: Universitary Hospital Vírgen de las Nieves in Granada, Hospital de León, Hospital La Paz and Hospital de Alcorcón in Madrid. The studies were approved by The Ethical Committees of the respective Hospitals, and informed consent was given by the patients. The three different studies in this thesis were carried out with different sample sets corresponding to oncology patients, mainly with prostate, ovarian, breast and lung cancer. The prostate samples were used to determine the expression pattern of cytochome P450 3A in normal and tumoral prostate tissue. Ovarian cancer samples were employed to study the association between miRNA expression, β-tubulin isoforms and response to taxanes treatment. And the samples from cancer patients treated with paclitaxel were used to identify genetic factors underlying the interindividual differences in neurotoxicity caused by paclitaxel. Below the specific details of each sample type are described:

1.1. Benign prostatic hyperplasias (BPH) and prostate cancer samples

These samples were collected in the Department of Pathology from the Universitary Hospital Vírgen de las Nieves in Granada with the collaboration of the Tumor Bank unit, coordinated by the Spanish National Cancer Research Centre (CNIO). The study included 14 benign prostatic hyperplasia (BPH) tissues and ten matched non-tumoral/tumoral prostate tissues; both frozen and paraffin embedded samples were available. Twenty-five additional tumoral prostate paraffin sections were obtained from the Department of Pathology from the Hospital de León. The specimens were selected from radical prostatectomies by an expert pathologist. Hematoxylin and eosin-stained sections were examined by a pathologist to determine the percentage of cancer cells in the tumor samples. The Gleason scores of the frozen samples were between five and seven and T stages of T2 or T3. The paraffin samples from the Hospital de León had Gleason scores between six and nine, with T stage T2 or T3 in all cases. In addition, seven liver samples were used for RT-PCR mRNA quantification.

1.2. Ovarian cancer samples

Seventy two ovarian cancer samples were collected in the Pathology Department, Hospital La Paz in Madrid. All patients received a platinum/taxane-based chemotherapy for at least 6 cycles and underwent a baseline CT scan and exploratory

laparotomy for diagnosis, staging, and debulking when feasible. The patients were classified according to the International Federation for 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 having achieved a CR after planned treatment, and all of them who were optimally debulked. In patients that after the treatment planned achieved a CR and did not accept a SLL, or whether this procedure was not feasible, and also in patients with a partial response, a second CT scan was performed one month after the first evaluation to confirm the response. Follow-up data were obtained by retrospective chart review. Progression-free survival (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. Hematoxylin and eosin-stained tissue sections were reviewed by an experienced pathologist and the selected samples included at least 80% of tumor cells and no large necrotic areas. All the samples were paraffin embedded. Most of the specimens were from serous cystadenocarcinomas (79%) with a mean follow-up of 43 months. The main clinical data associated to the samples is presented in Table 2.

Table 2. Characteristics of the 72 samples from patients with ovarian cancer.

Characteristics [a]	***No.***	***%***
Age at study entry (years) Mean ± SD	56.8 ± 10.8	
Histology		
Serous cystadenocarcinoma	57	79
Clear cell tumour	6	8
Endometrioid tumour	4	6
Mucinous cystadenocarcinoma	3	4
Mixed endometrioid-clear cell	1	1
Mixed endometrioid-serous	1	1
FIGO stage [b]		
I	3	4
II	5	7
III	54	75
IV	10	14
Grade of Differentiation		
1	8	11
2	24	33
3	39	54

[a] All patients included in the study were Caucasian females.
[b] FIGO = International Federation of Gynecology and Obstetrics.

For the analysis of response to treatment and survival, we used a homogenous series of patients: the subgroup of serous cystadenocarcinomas samples with Figo Stages III or IV (IIIa (12%), IIIb (16%), IIIc (54%) and IV (16%). Concerning the differentiation grade of these tumors, 61, 32 and 7% had grade 3, 2 and 1, respectively. The mean age of the patients was 56 years. Data on treatment response is presented in table 3.

Table 3. Response to treatment in patients with advanced (Figo stage III/IV) serous ovarian adenocarcinomas

Characteristics[a]	*No.*	*%*
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		
Yes	47	83
No	8	14
Unknown	2	3
Recurrence-free survival[b] (months)		
Mean ± SD	24.8 ± 25.9	
Exitus		
Yes	35	61
No	19	33
Unknown	3	5
Overall survival[c] (months)		
Mean ± SD	40.4 ± 26.3	

[a] All patients included in the study were Caucasian females.
[b] Recurrence-free survival (PFS) was defined as the time interval between the start of the treatment and the first confirmed sign of disease recurrence or progression.
[c] 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.

1.3. Samples from cancer patients treated with paclitaxel and treatment schedules

A total of 132 cancer patients treated with paclitaxel in the Oncology Unit of the Hospital Alcorcón were recruited between 2006 and 2008. Sixty-two cases were recruited in a retrospective basis (ie. paclitaxel administration started before the beginning of the study) and 56 prospectively from December of 2006 to March of 2008. Inclusion criteria were: histologically documented solid neoplasia; chemotherapy regimen including paclitaxel, age more than 18 years, life expectancy of ≥12 weeks; Eastern Cooperative Oncology Group (ECOG) performance status of 2 or less; no chemotherapy, hormonal therapy nor radiotherapy within 4 weeks before treatment; adequate contraception for women of childbearing potential; and adequate bone marrow, renal and hepatic function. Exclusion criteria were three or more previous chemotherapy regimens, hematologic malignancy and previous neuropathy caused by conditions such as alcoholism, diabetes mellitus or peripheral vascular disease.

DNA could not be obtained for six patients and key clinical data was not available for four patients. Four patients had clinical conditions that interfered with paclitaxel neurotoxicity evaluation and were excluded from the study: one patient had Spanish toxic oil syndrome, another had been diagnosed with multiple sclerosis, presented residual grade 2 neurotoxicity from a previous chemotherapy treatment and the last patient had a history of alcohol abuse. In total, 118 patients were included in the final analysis. Patient characteristics and chemotherapy regimens are summarised in Table 4.

Paclitaxel was supplied as a concentrated solution in a mixture of Cremophor EL and ethanol and was administered mainly as 1-hr intravenous infusion on weekly schemes and as 3-hr intravenous infusion on the first day of the 3-week cycles. Standard intravenous premedication consisted of dexamethasone (10 mg), dexclorfeniramina (5 mg), and ranitidine (20 mg), given 30 minutes before paclitaxel infusion.

Table 4. Characteristics of the 118 evaluable patients included in the study.

Characteristics[a]	*No.*	%
Age at study entry (years)		
Mean ± SD	60.7 ± 11.5	
(minimum-maximum)	29-87	
Gender		
Male	42	36
Female	76	64
Body weight (kg)		
Mean ± SD	66.2 ± 12.8	
(minimum-maximum)	46-122	
BSA (m^2)		
Mean ± SD	1.73 ± 0.18	
(minimum-maximum)	1.38-2.46	
Site of primary tumor		
Lung	39	33
Breast	38	32
Ovary	24	20
Uterus	6	5
Head and neck	4	3
Other[b]	7	6
Type of treatment		
Palliative	75	64
Adjuvant	39	33
Neoadjuvant	4	3
Chemotherapy[c]		
Paclitaxel 175 + Carboplatin	63	53
Paclitaxel 80	25	21
Paclitaxel 150 + Gemcitabine	7	6
Paclitaxel 90 + Bevacizumab	5	4
Paclitaxel 80 + Carboplatin	5	4
Paclitaxel 80 + Carboplatin + Trastuzumab	4	3
Paclitaxel 175 + Cisplatin	3	3
Paclitaxel 80 + Cetuximab	2	1
Paclitaxel 80 + Trastuzumab	2	1
Paclitaxel 175 + Lapatinib	1	1
FAC/FEC followed by Paclitaxel 80	1	1
Paclitaxel infusion time		
3h infusion	74	63
1h infusion	41	35
1h and 3h	2	2
4h infusion	1	1
Response to treatment		
Progressive disease	23	20
Stable disease	7	6
Partial response	37	31
Complete response	6	5
Unknown	6	5
Not assessable[d]	39	33

[a] All patients included in the study were Caucasians of European origin
[b] Other sites of primary tumor were: bladder, urinary tract, germinal, peritoneal and head and neck.

[c] Paclitaxel 80-90 mg/m² had mainly 1hr infusion and 150-175 mg/m² mainly 3hr infusion. All doses in mg/m² if not specified otherwise. The different treatments consisted on: paclitaxel 175 + carboplatin (paclitaxel 175; carboplatin AUC 6 /3 wks); paclitaxel 80 (paclitaxel 80 /weekly); paclitaxel 150 + gemcitabine (paclitaxel 150; gemcitabine 2500 /2 wks); paclitaxel 90 + bevacizumab (paclitaxel 1º, 8º, 15º d; bevacizumab 10 mg/kg 1º and 15º d /4 wks); paclitaxel 80 + carboplatin (paclitaxel 80 + carboplatin AUC 2 /weekly); paclitaxel 80 + carboplatin + trastuzumab (paclitaxel 80; carboplatin AUC 2; trastuzumab 2 mg/kg /weekly); paclitaxel 175 + cisplatin (paclitaxel 175; cisplatin 90 /3 wks) in one patient paclitaxel was administered ip; paclitaxel 80 + cetuximab (paclitaxel 80; cetuximab 250 /weekly); paclitaxel 80 + trastuzumab (paclitaxel 80; trastuzumab 2 mg/kg /weekly); paclitaxel 175 + lapatinib (paclitaxel 175 /3 wks; lapatinib1250 mg/d); FAC/FEC followed by paclitaxel 80 (FAC/FEC followed by paclitaxel 80 /weekly).
[d] Patients on adjuvant therapy were not assessable.

1.3.1. Neurotoxicity assessment and inclusion of relevant clinical data

The neurotoxicity developed by the different patients included in the study was evaluated with data collected from the medical records of the patients. Sensitive and motor neurotoxicity was graded according to the National Cancer Institute (NCI) Common Toxicity Criteria Version 2: sensory neuropathy grade 1 included numbness/ paresthesia in the feet, in grade 2 these symptoms were present in both fingers and feet, and grade 3 consisted of functional disabling numbness/ paresthesia. Motor neuropathy grade 1 was defined by weakness in feet, in grade 2 the symptoms were present in both extremities and patients with grade 3 had trouble walking. At the first day of each chemotherapy cycle an evaluation of neurologic symptoms (sensory symptoms such as numbness and tingling in fingers of hands and feet, difficulty feeling the shape of objects, and motor symptoms such as general weakness, trouble walking, and trouble buttoning buttons) and a physical and neurological examination was performed. The neurotoxicity developed and the modifications to the treatment regimen (dose reductions or suspensions) due to paclitaxel induced neurotoxicity are shown in Table 5. The accumulated dose of paclitaxel causing grade 2 neurotoxicity and the total accumulated dose of paclitaxel, and other relevant demographic and clinical data such as age, gender, body surface area (BSA), type of paclitaxel treatment, tumor characteristics, comorbidity factors and previous neurotoxic treatments, were included in an anonymous database.

Table 5. Neurotoxicity and treatment modifications caused by paclitaxel.

Neurotoxicity[a]	*n*	*%*
Sensory toxicity		
grade 0	43	36
grade 1	17	14
grade 2	44	37
grade 3	14	12
Motor toxicity		
grade 0	104	88
grade 1	7	6
grade 2	5	4
grade 3	2	2
Treatment modifications[b]		
No change	95	80
Reduction	9	8
Suspension	14	12

[a] Maximum neurotoxicity according to NCI Common Toxicity Criteria version 2.
[b] Modifications of the treatment due to paclitaxel induced neurotoxicity.

2. Isolation and quantification of RNA

Total RNA was isolated from frozen tissue using TRI reagent (Molecular Research Center Inc., Cincinnati, OH, USA) and from paraffin-embedded tissue samples using miRNeasy FFPE kit (Qiagen), according to the manufacturers' instructions. One microlitre of RNA was used to measure the concentration by Nanodrop ND-1000 (Wilmington, DE, USA) and the RNA quality was tested through 1% agarose gel electrophoresis.

3. Real time quantitative PCR (qRT-PCR)

3.1. qRT-PCR analysis of mRNAs

One microgram of the total RNA was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen) and an oligo dT14 primer following the manufacturer's instructions. mRNAs were quantified by real-time PCR with the Sequence Detection System 7900HT (Applied Biosystems), using specific primers and probes (Table 6) at a final concentration of 0.9 and 0.2 mM respectively, and the Universal Master Mix (PE Applied Biosystems). The amplification conditions consisted of an initial step at 95 ºC for 10 min, followed by 50 cycles of 15 s at 95 ºC and 1 min at

60 ºC. Standard curves were generated with serial 1/10 dilutions of cDNAs expressing high levels of the gene under study. Normalization was carried out with the internal standard β-glucuronidase (GUS). Negative controls were present in all series of PCRs and all assays were carried out in triplicates.

3.2. qRT-PCR of microRNAs

For microRNAs qRT-PCR, 25ng of total RNA were reverse transcribed using the miRCURY LNA™ First-strand cDNA Kit (Exiqon) 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 consisting on reaction mix without reverse transcriptase were included for the different microRNAs studied. Real-time quantitative PCR was performed with the Sequence Detection System 7900HT (Applied Biosystems) using the miRCURY LNA™ SYBR® Green Master Mix (Exiqon) following the manufacturer's instructions. The amplification conditions consisted of an initial step at 95 ºC for 10 min, followed by 50 cycles of 20 s at 95 ºC and 1 min at 60 ºC. Negative controls were included to all series of PCRs and all assays were performed in triplicates. The delta-delta Ct method was used for the calculation of the different amounts of mRNA {Livak, 2001 #326}. Normalization was carried out with the endogenous control 5S ribosomic RNA.

Table 6. Primers for mRNA qRT-PCR

Primers Taqman:	*Sequence:*
CYP3A4 FW	CATTCCTCATCCCAATTCTTGAAGT
CYP3A4 RV	CCACTCGGTGCTTTTGTGTATCT
Probe 3A4	FAM-CGAGGCGACTTTCTTTCATCCTTTTTACAGATTTT(C)-3BQ1
CYP3A5-total FW	GCTCGCAGCCCAGTCAATA
CYP3A5-total RV	AGGTGGTGCCTTATTGGGC
Probe 3A5-total	FAM-TGAAACCACCAGCAGTGTTCTTTCCTTCAC-3BQ1
CYP3A5 ex2 FW	GGGTCTCTGGAAATTTGACACAGAG
CYP3A5 ex3 RV	CTGTTCTGATCACGTCGGGATCT
Probe CYP3A5*1	FAM-ATGTGGGGAACGTATGAAGGTCAACTCCCT-3BQ1
CYP3A7 FW	AAGGGCTATTGGACGTTTGACA
CYP3A7 RV	ATCCCACTGGCCCGAAAG
Probe 3A7	FAM-TATTTATGACTGTCAACAGCCTATGCTGGCTATCA-3BQ1
CYP3A43 FW	AATACGAACATTGCTATCTCCAGCT
CYP3A43 RV	GCTTCTCACCAACATATCTCCACAT
Probe 3A43	FAM-TTCACCAGTGTAAAATTCAAGGAAATGGTCCC-3BQ1
GUS-FW	GAAAATATGTGGTTGGAGAGCTCATT
GUS-RV	CCGAGTGAAGATCCCCTTTTTA
Probe GUS	FAM-CCAGCACTCTCGTCGGTGACTGTTCA-3BQ1

4. Isolation and quantification of genomic DNA

DNA was isolated from frozen tissue samples using Qiagen DNeasy tissue kit (Qiagen, Valencia, CA, USA) and from peripheral blood using an automatic DNA extraction robot (Magnapure, Roche, Mannheim, Germany), according to the manufacturers' recommended protocols. For DNA extractions from saliva we used Oragene DNA Self-Collection Kits (DNA Genotek Ottawa, ON, Canada).

The concentration of DNA was quantified by PicoGreen (Invitrogen, Carlsbad, CA). For the standard curve, a series of dilutions of genomic DNA (Clontech, Mountain View, CA 94043 USA), giving a final DNA concentration from 5 to 160 ng/μl, were prepared in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). The standards and 2 μl of each sample were pipetted into a 96 well microplate (Falcon, BD Biosciences, San Jose, CA, USA) in duplicates. PicoGreen reagent was diluted in TE buffer according to the kit instructions and 195 μl of the mix was pipetted in the wells. After 5 minutes incubation in dark at room temperature, the Fluorescence was read at 520 nm after 480 nm excitation using DTX 800 Multimode Detector (Beckman Coulter, Fullerton, CA, USA).

5. Genotyping and sequencing

SNPs were genotyped using either KASPar SNP Genotyping System (Kbiosciences, Herts, UK) (Figure 6) or the Amplifluor SNPs Genotyping System (Chemicon International, Temecula, CA). The specific primers used for genotyping are shown in Table 7. Fifteen ng of DNA were used for the genotyping reactions, which were performed in duplicates. The sequence Detection System 7900HT (Applied Biosystems, Foster City, CA, USA) was used for fluorescence detection and allele assignment. Positive controls (i.e. DNAs with known genotypes) were included in all assays. In addition, 5% of samples representing the three different genotypes (i.e. wild type homozygotes, heterozygotes and variant homozygotes) were confirmed by other techniques and no deviation was observed between the two determinations.

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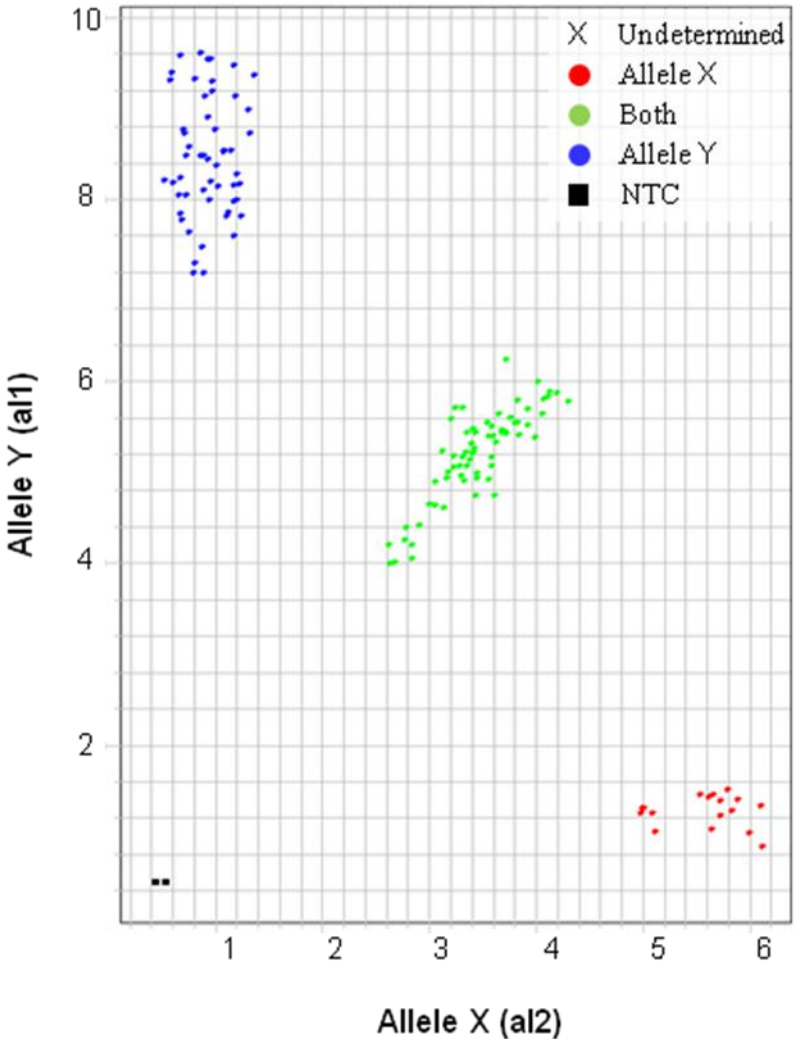

Figure 6. KASPar SNP Genotyping System. Genotypes were assigned by allelic discrimination with the Sequence Detection System 7900HT.

Direct sequencing by Sanger method was performed with sequencer 3730 from Applied Biosystems (Foster City, CA, USA)

Table 7. Primers for genotyping and sequencing.

Gene	dbSNP	Primer Allele 1	Primer Allele 2	Common primer	Genotyping method
CYP2C8	rs11572080,G>A	GAAGGTGACCAAGTTCATGCTTGAAC ACGGTCCTCAATGCTCT	GAAGGTCGGAGTCAACGGATTGAAC ACGGTCCTCAATGCTCC	TCTCCCTCACAACCTT GCGGAATTT	KASPar
CYP2C8	rs1058930, C>G	GAAGGTGACCAAGTTCATGCTATGTT AACAATCCTCGGGACTTTATC	GAAGGTCGGAGTCAACGGATTATGTT AACAATCCTCGGGACTTTATG	CTGTTGCTAATATCTT ACCTGCTCCATTT	KASPar
CYP2C8	rs1113129, G>C	GAAGGTGACCAAGTTCATGCTTGTCT CTTCTAACAGTATTTTCAAATAGG	GAAGGTCGGAGTCAACGGATTGTCTC TTCTAACAGTATTTTCAAATAGC	YCAGCAGAAGAAAGA ATTAGTGAGCTTTAA	KASPar
CYP2C8	rs7909236, G>T	GAAGGTCGGAGTCAACGGATTCTCC ATCATCACAGCACATTGGAAA	GAAGGTGACCAAGTTCATGCTCCATC ATCACAGCACATTGGAAC	GGATTGGAGCCCAGG TATTTTT	Amplifluor
CYP3A4	rs2740574, A>G	GAAGGTGACCAAGTTCATGCTGACA GCCATAGAGACAAGGGCAA	GAAGGTCGGAGTCAACGGATTACAG CCATAGAGACAAGGGCAG	CAAGTGGAGCCATTG GCATAAAATCTATT	KASPar
CYP3A5	rs776746, G>A	GAAGGTGACCAAGTTCATGCTATCTC TTTAAAGAGCTCTTTTGTCTTTCAA	GAAGGTCGGAGTCAACGGATTCTCTT TAAAGAGCTCTTTTGTCTTTCAG	GCCACCCAAGGCTTC ATATGATGAA	KASPar
ABCB1	rs2032582, G>T	GAAGGTGACCAAGTTCATGCTATTTA GTTTGACTCACCTTCCCAGC	GAAGGTCGGAGTCAACGGATTATATT TAGTTTGACTCACCTTCCCAGA	GGACAAGCAYTGAAA GATAAGAAAGAACTA	KASPar
ABCB1	rs1128503, C>T	GAAGGTCGGAGTCAACGGATTTCCT GGTAGATCTTGAAGGGC	GAAGGTGACCAAGTTCATGCTGTCCT GGTAGATCTTGAAGGGT	CCACAGCCACTGTTT CCAA	Amplifluor
ABCB1	rs1045642, C>T	GAAGGTGACCAAGTTCATGCTGGTG GTGTCACAGGAAGAGATC	GAAGGTCGGAGTCAACGGATTGGTG GTGTCACAGGAAGAGATT	ATGTATGTTGGCCTCC TTTGCT	Amplifluor
ABCB1	rs9282564, A>G	GAAGGTGACCAAGTTCATGCTATGAA AATGAAACAAGCTAGTTACCTTTTATT	GAAGGTCGGAGTCAACGGATTGAAAA TGAAACAAGCTAGTTACCTTTTATC	GGACCGCAATGGAGG AGCAAAGAA	KASPar
SLCO1B1	rs4149056, T>C	GAAGGTGACCAAGTTCATGCTCCAC GAAGCATATTACCCATGAACA	GAAGGTCGGAGTCAACGGATTCACG AAGCATATTACCCATGAACG	AAGGAATCTGGGTCA TACATGTGGATATA	KASPar
SLCO1B3	rs4149117, G>T	GAAGGTGACCAAGTTCATGCTTATGG GAACTGGAAGTATTTTGACAT	GAAGGTCGGAGTCAACGGATTATGG GAACTGGAAGTATTTTGACAG	CACTTACTATCCCATG AAGAAATGTGGTA	KASPar
SLCO1B3	rs7311358, A>G	GAAGGTCGGAGTCAACGGATTCTCA GATCTACATATCCAATATCCACGTAT	GAAGGTGACCAAGTTCATGCTTTCAG ATCTACATATCCAATATCCACGTAC	CTTTGCACTGGGATCT CTGTTT	Amplifluor
Sequencing primers:		**Forward primer**	**Reverse primer**		
CYP3A5	rs776746, G>A	TTATAAGGTGGTCTCAGCCAAT	CAGGGAGTTGACCTTCATACGTT		

6. Subcellular fractionation and protein concentration determination

To obtain microsomal fractions, tissues were homogenized with a glass homogenizer in four volumes of ice-cold 50 mM Tris–HCl, pH 7.4 containing 0.25 M sucrose and protease inhibitors. The resulting homogenate was centrifuged at 10,000 g for 20 min at 4ºC, followed by centrifugation at 100,000 g for 1 h at 4 ºC. The pellet was washed and resuspended in 0.1 M PBS, pH 7.4 with 10% glycerol and protease inhibitors (Roche). Protein concentration was measured by Bio-Rad protein assay (Bio-Rad laboratories) using bovine serum albumin (BSA) (Pierce) to create a standard curve with known concentrations of protein.

7. Antibodies

The following antibodies were used for protein detection: CYP3A5 antibody (ab22692, Abcam, Cambridge, UK), cytokeratin 34bE12 (34BE12, FLEX, DAKO, Copenhagen, Denmark), class I β-tubulin (clone SAP.4G5, Sigma-Aldrich, St. Louis, MO, USA); class II β-tubulin (clone 7B9, Covance, Emeryville, CA, USA); class III β-tubulin (clone TUJ-1, Santa Cruz Biotechnology, Heidelberg, Germany). Detection was performed with Envision Plus Detection System (Dako).

8. Immunoblot analysis

For the detection of CYP3A5, 50 μg of protein were loaded in each well, separated by 9% SDS-PAGE using the Mini-PROTEAN III electrophoresis cell (Bio-Rad) and transferred to polyvinylidene fluoride membranes (Immobilon-P Membrane, Millipore, Billerica, MA, USA). Equal loading of proteins was verified by Ponceau S staining with 0.1% Ponceau red in 5% acetic acid for 2 min. Ponceau S was washed out with PBS and the membranes were blocked with 5% non-fat dry milk in PBS for 1 h at room temperature. The membranes were then incubated with the primary antibody diluted 1:1500 following the manufacturer's instructions. After washing, the membranes were incubated with a goat anti-rabbit (DAKO) secondary antibody, and the corresponding horseradish peroxide signal was visualized using SuperSignal Femto substrate (Pierce, Rockford, IL, USA) and BiomaxLight membranes (Kodak). Protein content was determined from standard curves derived from samples that contained

known quantities of the specific protein under study. The detection limit under these conditions was of 0.025 pmol CYP3A5 protein/mg total microsomal protein.

9. 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), as described previously (Hardisson 2003). A hematoxylin and eosin-stained section of the array was reviewed to confirm the presence of morphologically representative areas of the original lesions.

10. Immunohistochemistry (IHC)

For the detection of CYP3A5 protein, immunohistochemical staining was performed by the DAKO Envision system (DAKO, Glostrup, Denmark) with a heat induced, antigen retrieval step. A 1:20000 and 1:1000 dilutions were used for the primary and secondary antibodies, respectively. Sections from the paraffin-embedded tissue were immersed in boiling 10 mM sodium citrate at pH 6.5 for 2 min in a pressure cooker and finally proteinase K was added for 10 min at room temperature. For the frozen tissues 5 mm sections were cut with a cryostat, dehydrated in 70% ethanol over night, fixed in acetone for 10 min, and stained with the same antibodies and dilutions used for the paraffin-embedded tissue, without antigen retrieval. Kidney samples were used to optimize the signal detection and the specificity of the signal was assured by following the same immunohistochemical staining procedure but without adding the primary antibody. The results were analyzed by two experienced pathologist.

For the detection of the β-tubulin isotypes I, II and III IHC was performed on 4-µm sections of formalin-fixed, paraffin-embedded tissue microarrays. 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 50ºC for 45 minutes in an autoclave. Endogenous peroxidase and non-specific antibody reactivity was blocked with peroxidase blocking reagent (Dako) at room temperature for 15 minutes. The sections were then incubated for 60-90 minutes at 4ºC with the following antibody

dilutions: class I β-tubulin (dil. 1:100), class II β-tubulin (dil. 1:100), class III β-tubulin (dil. 1:200). Immunoreactivity was scored by estimating the percentage of tumor cells with cytoplasmic immunostaing. Labeling frequency was scored on a three-tiered system as absent, low expression (≤75%) and high expression (>75% of tumor cells showing immunoreactivity).

11. Statistical analysis

All statistical analyses were carried out using SPSS software package version 17.0 (SPSS, Inc., Chicago, IL). Nominal two-sided P-values less than 0.05 were considered statistically significant.

The association between the expression levels of the miR-200 microRNAs was determined by Pearson coefficient. An association between microRNAs expression and continuous demographic variables (such as age) was also studied by Pearson coefficient. For categorical variables (such as treatment response, pathological response, survival and relapse, and tumoral characteristics), since the tumoral miR-200 family content followed a normal distribution (Kolmogorov–Smirnov test), Student t-tests were used applying Welch correction when the standard deviations differed significantly between groups. Protein expression of β-tubulin II and III was used as a categorical binary variable (low expression or high expression) using 75% of positive cells as the cut-off. The response was divided into two categories: patients with complete response and those with partial response, 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 the χ^2 test, Fisher's exact test and Student's T-test when appropriate. To further analyse response to treatment (dichotomic clinical and pathological response) with respect to microRNA expression a logistic regression model was applied and the Odds Ratio (OR) was estimated. A multivariate logistic regression model was used to adjust for relevant clinicopathological variables.

Kaplan-Meier analysis (log-rank test on 2 degrees freedom) was used to evaluate the effect of microRNA expression on disease outcome for both recurrence-free and overall survival. In addition, univariable and multivariable Cox regression analyses were performed, the latter including T-stage, grade of differentiation and debulking status as covariates.

For the paclitaxel neurotoxicity study we estimated, based on a sample size of 120 patients, with a prevalence of neurotoxicity of 50%, that we had over 80% power at an alpha level of 0.05 to detect per-allele risk ratios (RR) of at least 2 for allele frequencies above 5%. The power was less for protective effects (RR>0.50) associated with the minor allele, although remained above 80% for allele frequencies above 15%. Associations between genotypes and risk of paclitaxel neurotoxicity were first tested for individual polymorphisms using Kaplan-Meier analysis (Kaplan and Meier 1958) (log-rank test on 2 degrees freedom), modeling the cumulative dose of paclitaxel up to the development of grade 2 neurotoxicity. Patients with no or minimal adverse reaction (grade 0/1) were censored at total cumulative dose. Associations with neurotoxicity were also tested in this way for age (quartiles), gender, BSA (quartiles), paclitaxel treatment regimen (weekly 80-90 mg/m^2 *versus* every 3 weeks scheme 150-175 mg/m^2), previous neurotoxic treatments, comorbidity factors (diabetes mellitus, alcohol intake, cardiopathy, nephropathy, etc.), retrospective *versus* prospective cases and type of treatment (palliative *versus* adjuvant or neo-adjuvant). Associations with cumulative dose of paclitaxel up to the development of grade 2 neurotoxicity were also evaluated for each polymorphism using univariable and multivariable Cox regression under a log-additive model, the latter including age and treatment regimen as covariates, since they were found to be independently associated with neurotoxicity. We also evaluated a model including all SNPs for which individual associations were observed, in which the variants associated with increased metabolic activity acted in a log-additive fashion, both within and between SNPs. This was done by analyzing the number of such variants carried as a continuous variable.

MATERIALES & METODOS

1. Pacientes

Las muestras de los pacientes de cáncer utilizados en esta tesis doctoral fueron recogidas gracias a una estrecha colaboración con los siguientes hospitales españoles: Hospital Virgen de las Nieves en Granada, Hospital La Paz, Hospital de León y Fundación Hospital de Alcorcón en Madrid. Los estudios fueron aprobados por el correspondiente comité ético de cada hospital y las muestras se recogieron tras la firma de un consentimiento informado. Los tres estudios que componen esta tesis se llevaron a cabo con pacientes oncológicos distintos: 1. Para determinar la expresión de los citocromos P450 3A (CYP3A) en tejido próstatico normal y tumoral, trabajamos con 14 muestras de hiperplasia benigna de próstata (BHP), y con 10 muestras pareadas de próstata normal/ tumoral. 2. Para estudiar la posible asociación entre la expresión de microARNs (miARNs), la expresión de las isoformas I, II y III de β-tubulina y la respuesta al tratamiento con taxanos, se utilizaron 72 muestras de cáncer de ovario. 3. Para identificar variantes genéticas asociadas al riesgo de desarrollar neurotoxicidad por paclitaxel, se utilizaron 118 muestras de ADN de pacientes tratados con paclitaxel que tenían asociados los datos demográficos y clínicos más importantes en bases de datos codificadas.

2. Técnicas relacionadas con ARN

El ARN total se aisló del tejido congelado y del tejido parafinado mediante los kits comerciales TRI reagent (Invitrogen) y miRNeasy FFPE kit (Qiagen). La concentración de ARN se cuantificó mediante Nanodrop ND-1000. Para obtener ADN complementario se retrotranscribió un microgramo de ARN mediante Superscript II Reverse Transcriptase y oligo dT14. Las cantidades de ARN mensajero de genes específicos fueron cuantificadas mediante RT-PCR a tiempo real (qRT-PCR), usando *primers* y sondas específicas (Tabla 6). Para la cuantificar la expresión de los miARNs hsa-miR-141, hsa-miR-200a, hsa-miR-200b, hsa-miR-200c, hsa-miR-429 y del 5S rRNA se utilizó qRT-PCR partiendo de un total de 25 ng de ARN total siguiendo las instrucciones de los kits de EXIQON: miRCURY LNA™ First-strand cDNA Kit y un conjunto de primers de miRCURY LNA™.

3. Técnicas relacionadas con ADN

El ADN genómico fue aislado a partir de muestras de saliva, sangre o tejido congelado de pacientes mediante: Oragene DNA Self-Collection Kits (DNA Genotek), extracción automática de ADN (Magnapure) y Qiagen DNeasy tissue kit, respectivamente. La concentración de ADN se cuantificó mediante Picogreen (Invitrogen). La genotipación de polimorfismos se llevó a cabo utilizando las técnicas:

Amplifluor SNPs HT Genotyping (Chemicon International) o KASPar SNP Genotyping System (Kbiosciences) utilizando *primers* específicos. Estos datos sirvieron para crear una segunda base de datos que recogía los datos genéticos de los pacientes.

4. Técnicas relacionadas con proteína

50 μg de proteína microsomal aislada a partir de tejido prostático fueron utilizados para *Western blot.* Las proteínas se separaron mediante electroforesis con el sistema SDS-PAGE, y se transfirieron a una membrana PVDF de Millipore. Tras las incubaciones con los anticuerpos primario y secundario se detectaron las proteínas de interés mediante el sistema SuperSignal Femto substrate (Pierce) y BiomaxLight membranes (Kodak).

Las técnicas de immunohistoquímica se llevaron a cabo utilizando anticuerpos apropiados en cortes de tejidos parafinados, utilizando el sistema de DAKO Envision system (DAKO).

5. Análisis estadístico

Para el analisis estadístico se utilizó el programa de estadística SPSS versión 17.0. Se llevaron a cabo estudios estadísticos para determinar qué variables biológicas se asociaban significativamente la con respuesta/ toxicidad de los pacientes mediante la comparación de los datos clínicos y los genotipos/ niveles de expresión. Mediante análisis de Kaplan-Meier y regresión de Cox se estudió la asociación entre la expresión de microRNAs y la respuesta a tratamiento y la supervivencia. La asociación entre el nivel de expresión de los microRNAs fue determinado por coeficiente de Pearson, igual que asociaciones con otras variables continuas. Para variables categóricas (como respuesta, respuesta patológica, características del tumor) se utilizó el test de t-Student y regresión logística. Las asociaciones entre los genotipos y la neurotoxicidad del paclitaxel fueron estimados teniendo en cuenta la dosis acumulada de paclitaxel hasta el desarrollo de neurotoxicidad de grado 2 y utilizando los análisis de Kaplan-Meier y la regresión de Cox. La regresión de Cox se llevó a cabo usando un modelo aditivo con análisis univariantes y multivariantes, este último incluyendo la edad y el régimen terapeútico como covaraibles, ya que éstas se asociaban de forma independiente con la neurotoxicidad.

RESULTS

Results, part I

Cytochrome P450 3A5 is highly expressed in normal prostate cells but absent in prostate cancer

1. Cytochrome P450 3A5 is highly expressed in normal prostate cells but absent in prostate cancer

CYP3A enzymes metabolize testosterone and DHEA to less active compounds and it has been suggested that *CYP3A* polymorphisms could modify prostate cancer risk. Case-control studies aimed to study this association have obtained mostly positive but also contradictory results. However, the biological base for this connection remains uncertain, since the expression of these enzymes in the prostate is uncharacterized. This led us to investigate the expression of the four *CYP3A* genes in prostate normal tissue. We used quantitative RT-PCR to measure the CYP3As mRNA content in 24 non-tumoral prostate tissues and compared it with the expression of a pool of livers, which is the tissue with the highest CYP3A content.

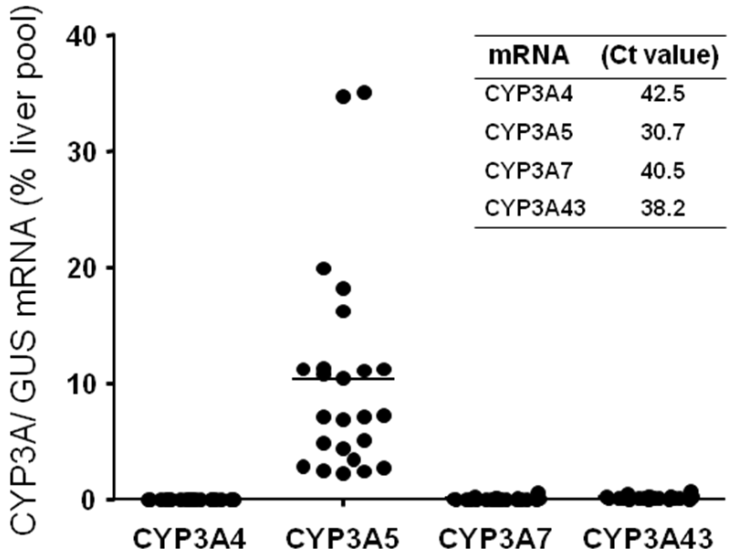

Figure 7. CYP3A5 mRNA is expressed at relevant levels in prostate normal tissue. The mRNA content of CYP3A4, CYP3A5, CYP3A7, and CYP3A43 was measured by quantitative RT-PCR, as described in Materials and methods section, in 24 prostate tissues. For CYP3A5 the primers that are used measured the total amount of CYP3A5 mRNA (correctly plus alternatively spliced mRNA). The amount of CYP3A mRNA was normalized with the GUS mRNA content in each sample. The quantification was performed using a pool of seven liver cDNAs and the results are expressed as percentage of the liver expression. The average number of PCR cycles needed to amplify each gene above the threshold (Ct) is shown in the insert.

1.1. CYP3A5 is expressed at high levels in normal prostate tissue

We found that CYP3A5 mRNA content in prostate was the highest (10% of hepatic levels), while CYP3A4, CYP3A7, and CYP3A43 mRNA content was much lower (0.0004, 0.05, and 0.15% of the liver levels, respectively), and that more than seven PCR amplification cycles separated the mRNA content of CYP3A5 from the other CYP3A enzymes (Figure 7). The prostate CYP3A5 mRNA content ranged from 7.9 to 69% of the hepatic levels, depending on the liver used for comparison. For CYP3A4 and CYP3A7 the maximum values were below 1%, while for CYP3A43, due to a very low CYP3A43 content in one of the livers up to 5.4% could be observed. Therefore, the only CYP3A enzyme expressed at biological relevant levels in the prostate was CYP3A5.

To determine whether CYP3A5 expression could be influenced by the localization within the human prostate gland (peripheral, central, and transitional zone), we compared the expression of the 14 BPH, which all correspond to the transitional zone, with 10 non-tumoral samples from the peripheral zone. However, we did not find significant differences in the CYP3As expression between the prostate samples, suggesting that CYP3A5 expression is homogeneous within the glandular regions of the prostate.

1.2. CYP3A5 expression is influenced by CYP3A5*3 polymorphism

Since CYP3A5 showed a 16-fold inter-sample variability in the 24 prostate cases studied, we decided to investigate whether polymorphisms in the *CYP3A5* gene could be influencing its expression in the prostate. In the liver, CYP3A5 expression is influenced to a large extent by *CYP3A5*3* polymorphism which, with a 90% allele frequency in Caucasians, creates a cryptic consensus splice site in intron 3. *CYP3A5*3* results mainly in splice variant mRNAs that contain a premature termination codon (amino acid 102) and only a small amount of correctly spliced CYP3A5 mRNA and protein is produced. Thus, to determine the influence of *CYP3A5*3* in prostate, we performed CYP3A5 qRT-PCR analysis using liver samples with different *CYP3A5* genotypes and quantified both the correctly spliced and the total (correctly spliced plus alternatively spliced) CYP3A5 mRNAs: a pool of two *CYP3A5*1/*3* liver cDNAs were used to generate the standard curve and a pool of four *CYP3A5*3/*3* liver cDNAs were included for quantification. We found that, similarly to liver, *CYP3A5*3/*3* prostate samples had lower CYP3A5 mRNA content than those *CYP3A5*1/*3* (3.3- and 13-fold

lower total and correctly spliced CYP3A5 mRNA in prostate compared with 2.8- and 10-fold difference in the liver samples respectively; Figure 8). The CYP3A5 total mRNA content of the prostate samples was 25% of the average amount in liver samples with the same *CYP3A5* genotype (Figure 8A upper panel), and 15% for correctly spliced mRNA (Figure 8A lower panel).

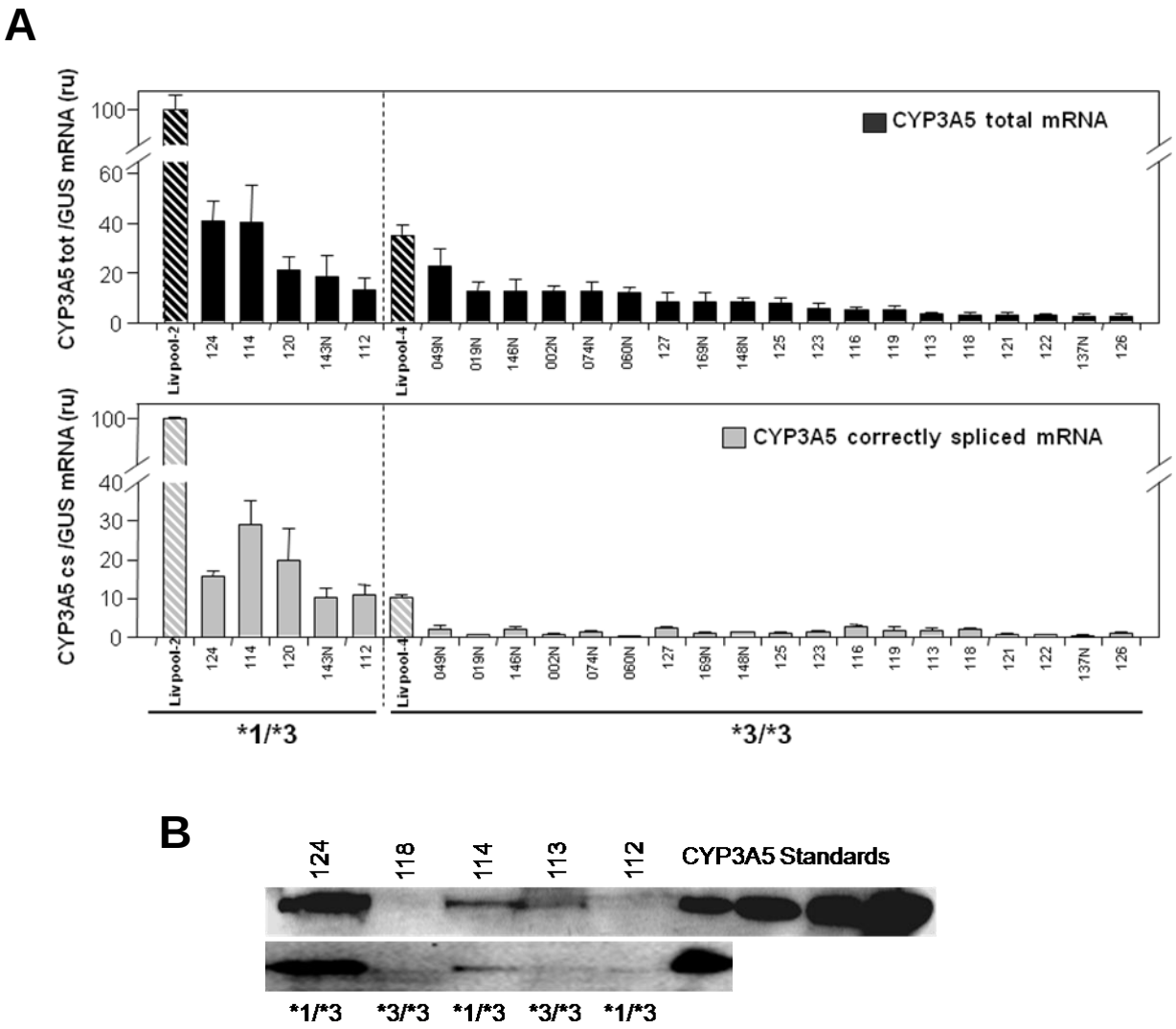

Figure 8. CYP3A5 mRNA and protein content in human prostate. (A) The amounts of total and correctly spliced CYP3A5 mRNA were quantified in 24 prostate samples and two liver pools using specific primers: the black bars correspond to total (tot) CYP3A5 mRNA while the gray bars correspond to correctly spliced (cs) mRNA. The *CYP3A5* genotype of the samples is shown below as '*1/*3' (five prostate samples and one pool of two livers) and '*3/*3', (19 prostate samples and 1 pool of 4 livers). The liver pools are shown with dashed bars. Relative units (ru); liver pool (Liv pool). (B) CYP3A5 protein expression was analyzed by western blotting in BPH microsomes (samples 112, 113, 114, 118, and 124) using a CYP3A5-specific antibody and commercial CYP3A5 Supersomes were used as standards for quantification. Two independent experiments are shown. The CYP3A5 genotype of each prostate sample is indicated below.

To further characterize the expression of CYP3A5 in prostate, we performed a western blot analysis using a CYP3A5-specific antibody. As shown in Figure 8B, CYP3A5 protein was detected in only some BPH microsomal fractions. Although there was not a strong correlation between CYP3A5 mRNA and protein content, the strongest signals corresponded to *CYP3A5*1/*3* samples. The amount of CYP3A5 protein in sample 124 (with the highest expression) was of 0.15 pmol CYP3A5 per mg of total microsomal protein, which is much lower than CYP3A5 hepatic levels. However, this data should be referred to the prostate cells expressing CYP3A5. In the liver the hepatocytes, which represent the major part of the cells in this organ, express the CYP3A enzymes, but no information is available about the fraction of prostate cells expressing CYP3A5, thus, it was important to determine CYP3A5 localization.

1.3. CYP3A5 is expressed exclusively in the basolateral cells of the prostate

IHC was used to determine the localization of CYP3A5 in the prostate. As shown in Figures 9A and 9B, both in the frozen and paraffin-embeded tissues corresponding to the 24 non-tumoral samples analyzed, CYP3A5 staining was localized exclusively in the basolateral cells, specifically in the cytoplasmatic region, as expected for a microsomal protein. In contrast, stromal and luminal cells did not express CYP3A5. The prostate basolateral cell marker cytokeratin 34bE12 was used to confirm CYP3A5 basal localization (Figure 9F). This data implies that since CYP3A5 protein and mRNA quantifications were performed using extracts from total prostate tissue (including stromal, luminal, and basolateral cells), but only the basolateral cells contribute to CYP3A5 expression, the actual CYP3A5 levels of the basolateral cells must be higher than the calculated 15–25% of hepatic mRNA levels and 0.15 pmol/mg microsomal protein.

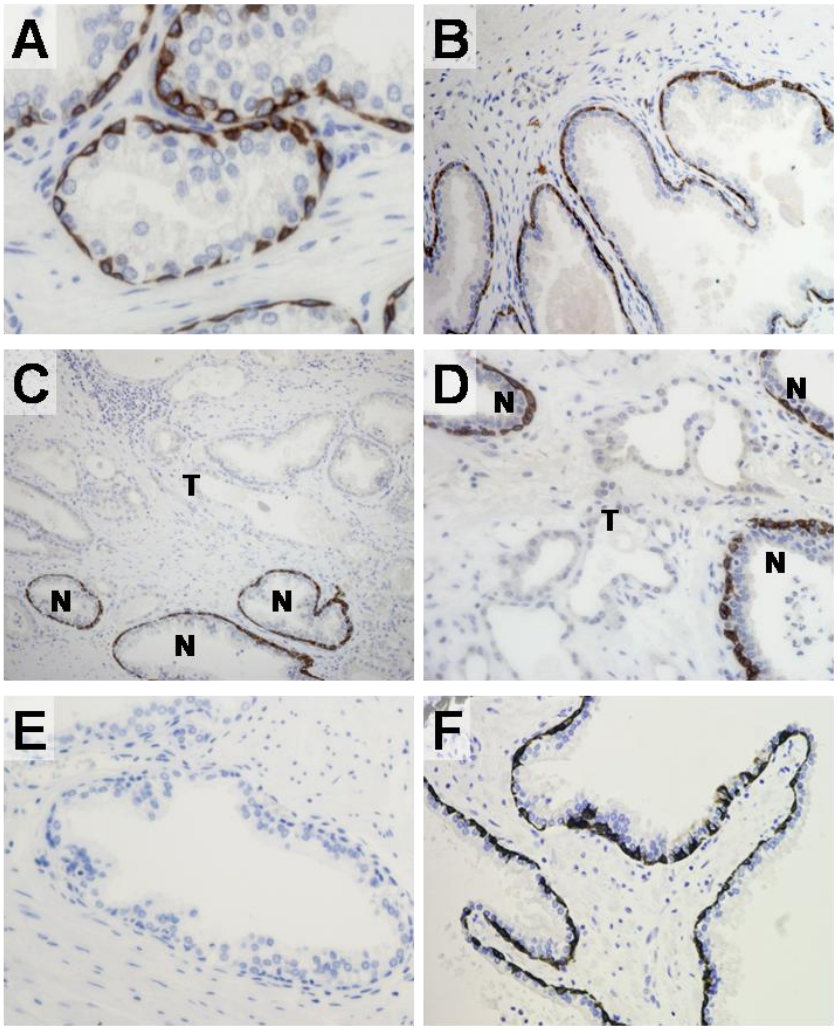

Figure 9. CYP3A5 protein detection by immunohistochemistry in prostate non-tumoral and tumoral tissue. Immunohistochemical staining of CYP3A5 was performed in paraffin sections as described in Materials and methods section. In the non-tumoral tissue (A and B) CYP3A5 was localized in the prostate glands, specifically in the basolateral cells of CYP3A5*1/*3 (143N, A) and CYP3A5*3/*3 (169N, B) samples. Immunohistochemical staining of CYP3A5 in paraffin sections of tumoral prostate samples (C and D) showed that CYP3A5 was absent in the tumoral areas (T) and CYP3A5 signal was localized only in the surrounding normal tissue (N). The tumor samples analyzed were: 143T (C), 169T (D). (E) shows the same immunohistochemical staining procedure in 143N but without CYP3A5 antibody and (F) corresponds to an IHC staining using the basolateral marker cytokeratin 34bE12. Original magnification !20, except for A with X10 and for B with X40.

The effect of *CYP3A5*3* was not evident with the standard IHC conditions. However, when using lower amounts of the antibody, a difference in the intensity of the CYP3A5 signal could be appreciated between *CYP3A5*1/*3* and *CYP3A5*3/*3* samples (Figure 10).

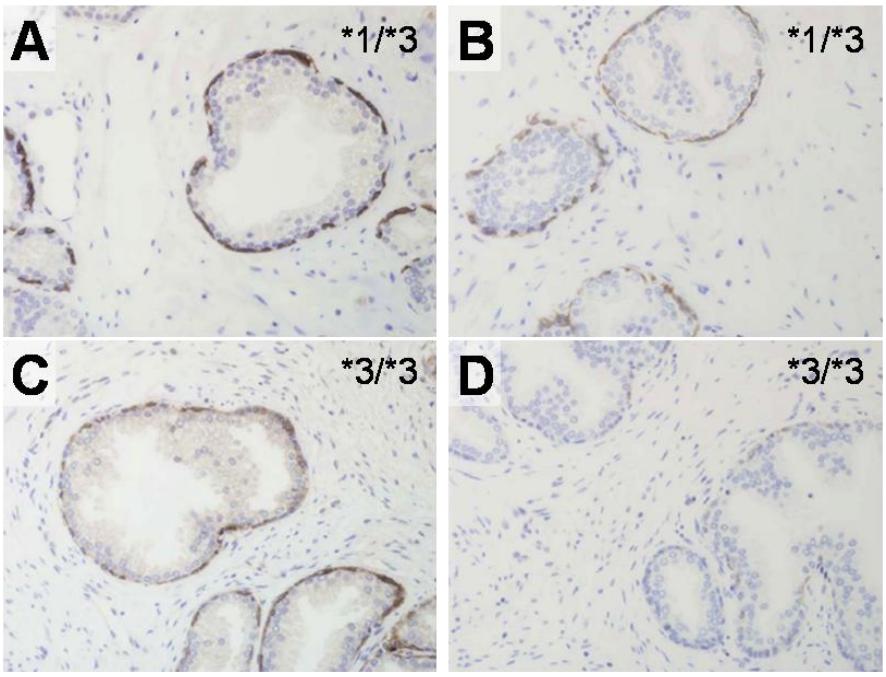

Figure 10. CYP3A5*3 affects protein expression. Immunohistochemical staining of CYP3A5 was performed in paraffin sections corresponding to *CYP3A5*1/*3* (143N**, A** and **B**) and *CYP3A5*3/*3* (169N**, C** and **D**) genotypes. The CYP3A5 antibody dilutions used were 1/20 000 (A and C) and 1/100 000 (B and D).

1.4. Tumoral prostate tissue lacks CYP3A5 expression

We then investigated the expression of CYP3A5 in tumoral prostate tissues. We examined CYP3A5 protein content in ten matched non-tumoral/tumoral samples by IHC, finding that in all of them there was a complete lack of CYP3A5 protein in the tumoral areas of the tissue and that only the surrounding areas containing non-tumoral normal glands showed CYP3A5 staining (Fig. 9C and D). The lack of CYP3A5 expression in the tumor is consistent with its basolateral localization, since prostate tumors lack basal cells. To confirm this finding, we analyzed the expression of the basolateral marker 34bE12 and found it absent, similarly to CYP3A5, in the prostate cancer regions. The lack of CYP3A5 expression in the tumoral areas was confirmed in 25 additional prostate cancer samples.

Analysis by qRT-PCR of the ten matched non-tumoral/tumoral samples confirmed the IHC results, and we found that CYP3A5 mRNA was significantly lower in the tumoral tissue (in average 61- and 19-fold difference for total and correctly spliced CYP3A5 mRNA respectively; Table 8). The detection of some CYP3A5 mRNA by qRT-

PCR in the tumoral samples can be easily explained by the contribution of the surrounding contaminating non-tumoral tissue present in the tumoral blocks (see Figure 9C and D). Consequently, the CYP3A5 RT-PCR and the IHC data are in full agreement.

Table 8. CYP3A5 mRNA content was decreased in the tumoral samples when compared to the matched non-tumoral tissue.

Prostate Sample	*Fold decrease [a] (total mRNA)*	*Fold decrease (c.s. mRNA [b])*
002	59	15
019	90	11
049	248	54
060	6	1
074	89	8
137	2	4
143	34	23
146	4	6
148	72	63
169	9	4
Mean	***61***	***19***

[a] Fold decrease of CYP3A5 mRNA in the tumoral tissue when compared to matched non-tumoral tissue.
[b] Correctly spliced mRNA (c.s. mRNA).

Results, part II

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

2. miR-200 family controls β-tubulin III expression and is associated with treatment outcome in ovarian carcinoma patients

Over-expression of β-tubulin III in tumor cells has been proposed to contribute to the lack of efficacy for the microtubule-binding drugs. However, the molecular mechanism(s) underlying the up-regulation of β-tubulin III are still unknown. Recently, *in vitro* studies have suggested that class III β-tubulin protein expression could be influenced by the microRNA miR-200c, which forms part of the miR-200 family. The important role that the miR-200 family plays in metastasis and the high conservation among β-tubulin isotype functions and genetic structure, led us to explore the regulation of β-tubulin isotypes by miR-200 family members.

2.1. Predicted binding sites for miR-200b/c/429 in the 3´UTR of β-tubulins and immunohistochemical expression of β-tubulin isotypes I, II and III

In silico analysis of the eight different human β-tubulin genes (http://www.targetscan.org/) predicted binding sites for *miR-200b/c/429* in the 3'UTR of class I, II and III β-tubulins, while no binding was predicted for the rest of human β-tubulin isotypes (Figure 11). This microRNA binding site was broadly conserved among vertebrates for class I and III β-tubulins, but poorly conserved for class II. The *in silico* analysis did not predict miR-141 and miR-200a binding due to one nucleotide difference in the seed sequence (Figure 11), but there are evidences of common targets for the whole miR-200 family.

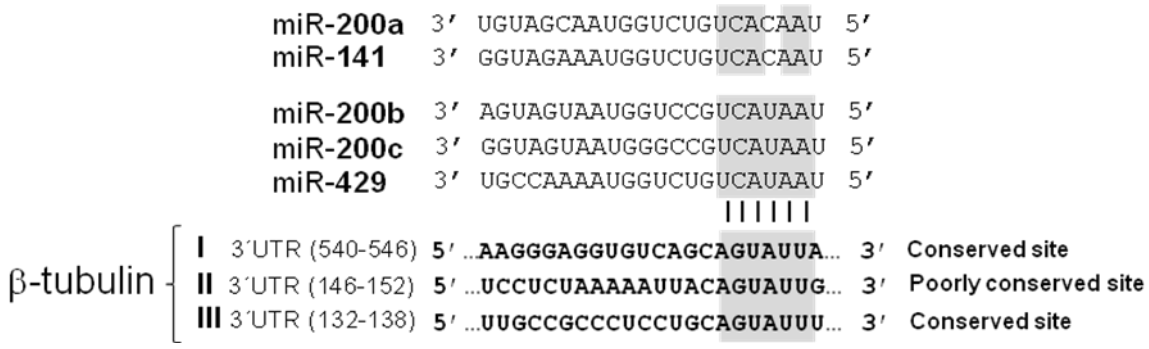

Figure 11. Predicted miR-200b/c/429 binding sites in the 3'UTR of β-tubulin isotypes I, II and III. The six base pairs seed region common for miR-200b/c/429 is shown together with the predicted binding sites in the 3'UTR regions of β-tubulin isotypes I, II and III. The miR-200a and miR-141 recognition sequence is also shown including the nucleotide change in the seed sequence.

We then determined the protein expression of class I, II and III β-tubulins in 72 ovarian cancer samples by IHC analysis (Figure 12). The protein staining showed substantial differences for the different isotypes: β-tubulins II and III exhibited important inter-sample differences, while class I protein expression showed no variation among the cases studied. As shown in Figure 13A, all samples exhibited a very strong class I β-tubulin staining; class II protein was absent in 46 tumors, while 15 had low and 6 cases a high protein expression (22 and 9% of the tumors, respectively). Class III protein was absent in 34 cases, while 29 had low and 8 had a high protein expression (41 and 11%, respectively). The expression of class II and III β-tubulins were mutually exclusive events, with samples exhibiting a high β-tubulin III content lacking isotype II expression and *vice versa* (Figure 13B).

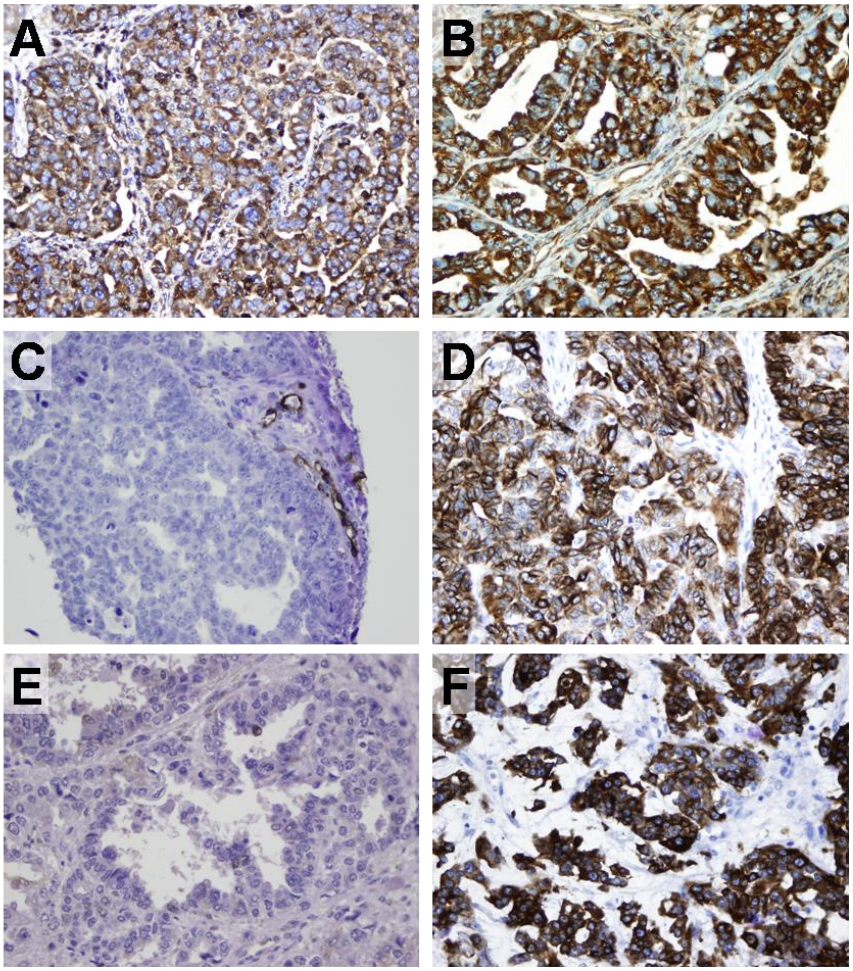

Figure 12. Protein expression of β-tubulin isotypes I, II and III in ovarian tumors. Immunohistochemical staining was performed in paraffin sections as described in Materials and Methods section. Isotype I exhibited a high expression in all cases analyzed with minimal differences among samples (A) and (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 cystadenocarcinomas.

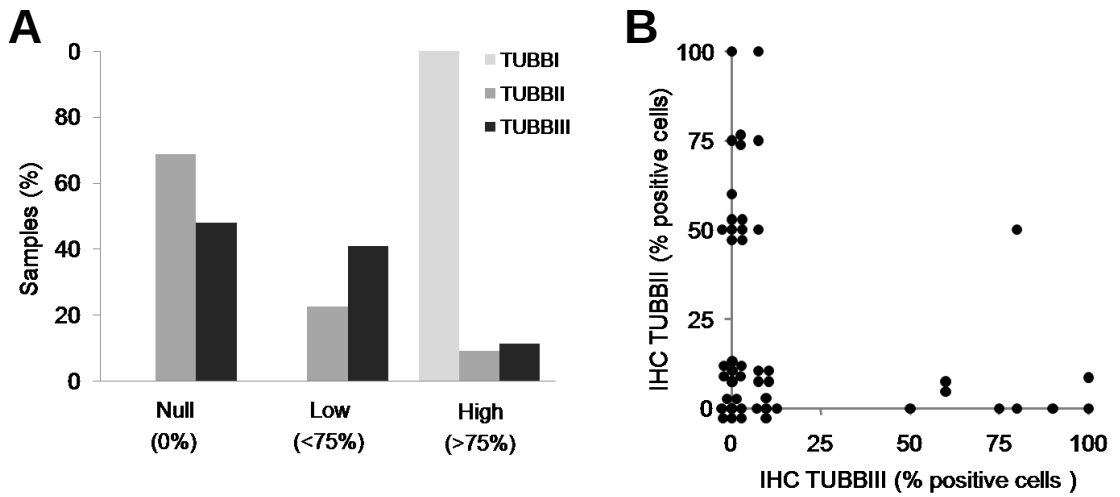

Figure 13. Distribution of ovarian tumors according to β-tubulin isotypes I, II and III protein expression. (A) All the tissues had a strong isotype I staining, while isotypes II and III exhibited substantial differences in protein content among the tumors. Null, indicates lack of staining, low, indicates that less than 75% of the cells scored positive for staining and High, indicates that more than 75% of cells exhibiting positive staining. β-tubulin isotypes I, II and III correspond to light grey, dark grey and black bars, respectively. (B) Mutual exclusive protein expression of β-tubulin isotypes class II and III.

2.2. miR-200 expression determines tumoral TUBBIII protein content

To study whether the miR-200 family could regulate β-tubulin isotypes I, II and III, we measured the expression of these microRNAs in the 72 ovarian cancer samples. The expression levels varied among the samples: the highest expression corresponded to miR-200c, then, miR-200b, miR-200a and the lowest expression to miR-429 and miR-141, which exhibited similar expression levels (Figure 14). The expression of the five microRNAs correlated, with miR-141/miR-200a and miR-200a/miR-200b showing the highest level of correlation and miR-429 the lowest (Table 9).

Table 9. Correlation between the expression of the miR-200 family members in 72 ovarian carcinomas. Pearson correlation coefficients are shown together with P values in brackets. Significant P values are shaded in light blue and extremely significant values in dark blue.

	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)	

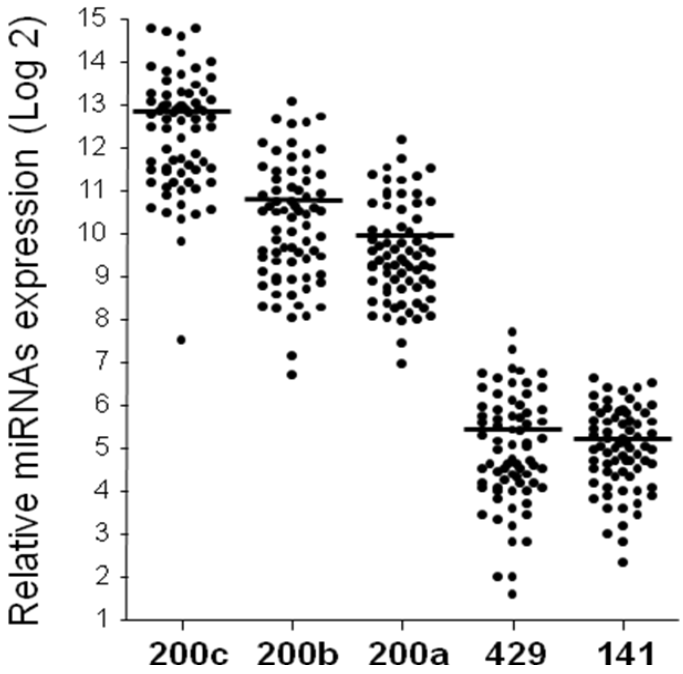

Figure 14. miR-200 family expression in ovarian tumors. The microRNA content of the five miR-200 members (miR200c/200b/200a/429/141) was measured by qRT-PCR, as described in Materials and Methods section in 72 ovarian tumors. The amount of microRNA was normalized with the 5S mRNA content in each sample. The quantities are expressed in relative units (ru).

We then determined whether the miR-200 family could play a part in the regulation of the β-tubulin isotypes I, II and III expression. We found a statistically significant association between class III β-tubulin protein expression and the tumoral content of all miR-200 members (Figure 15). In this way, the ovarian tumors with low miR-200 expression exhibited high class III protein content, suggesting that the absence of these microRNA in the tumors results in lack of class III β-tubulin degradation and accumulation of high levels of the protein. The most significant 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 and miR-200 family expression.

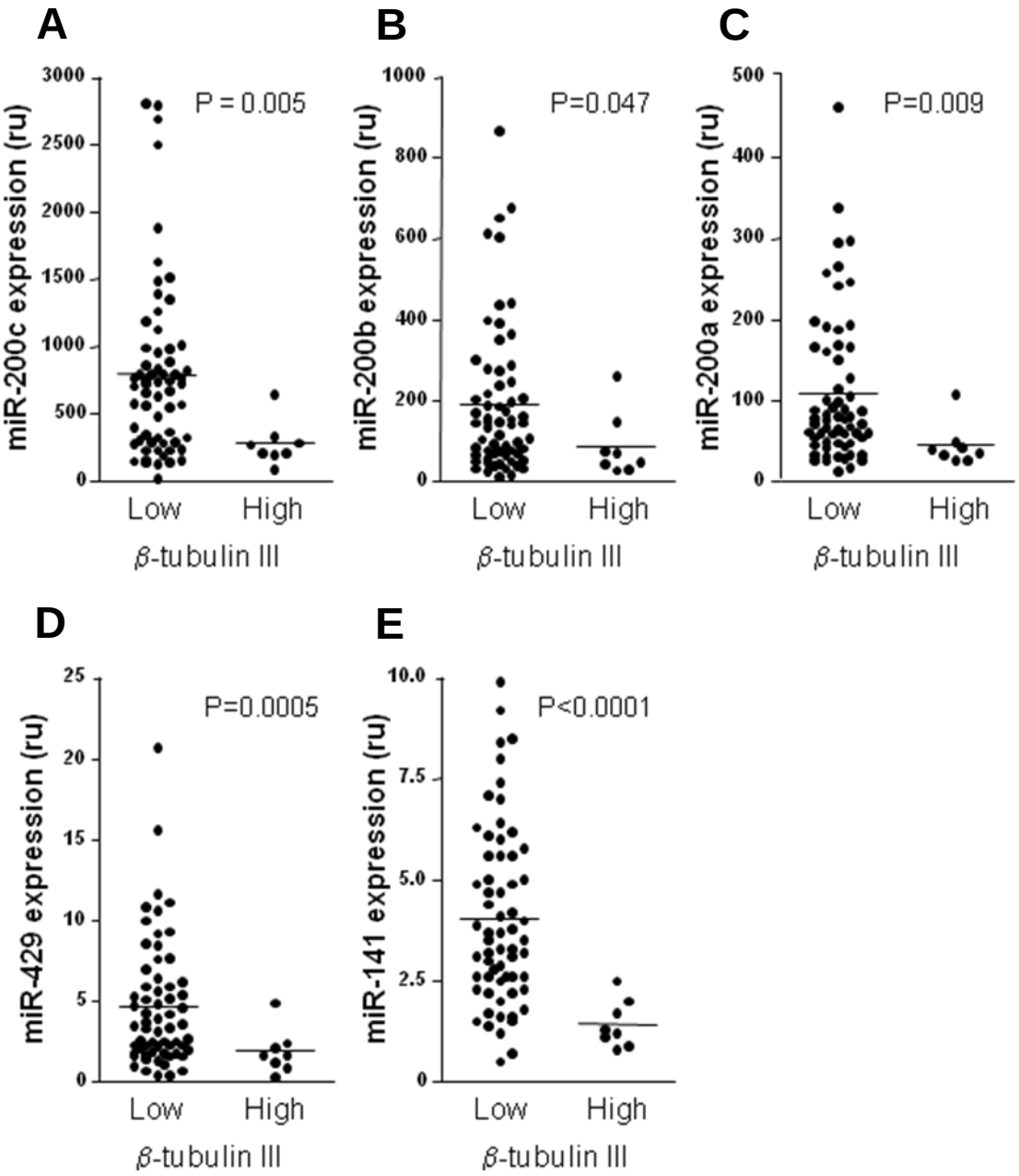

Figure 15. Tumors with high β-tubulin isotype III protein content have significantly decreased miR-200 expression. Samples with high isotype III expression (more than 75% positive cells) showed a significantly lower A) miR-200c (p=0.005), B) miR-200b p=0.047), C) miR200a p=0.009), D) miR-429 p=0.0005) and E) miR-141 p<0.0001) expression compared to samples with low isotype III expression. microRNAs are shown in the figure according to expression levels (A-E). To express the microRNAs content as whole numbers, their expression was multiplied by 100 and expressed as relative units (ru)

2.3. Response to taxane-based treatment and progression-free survival are associated with miR-200 expression

The ovarian cancer patients included in this study were all treated with the same protocol (6 cycles of platinum/taxane-based chemotherapy). However, since clinical stage and tumor type can greatly influence treatment outcome, to study whether miR-200 expression could impact response to treatment, we selected a homogenous subgroup of patients: those with advanced stages (III and IV) and serous cystadenocarcinoma. In total there were 57 patients with these characteristics. We found a statistically significant association between miR-200c expression and response to treatment (p=0.0027 with t-test; OR=0.70, 95%CI=0.50-0.98, P=0.037 in multivariate analysis, Table 10). The patients that did not achieve a complete clinical response had lower miR-200c levels than patients with complete response. A significant association was also found between miR-200c and pathological response in the univariate analysis (P =0.045), although it did not reach significance in the multivariate analysis (OR=0.69, 95%CI=0.45-1.07, P=0.094). Higher expression of miR-200c was associated with protection against recurrence (OR=0.86, 95% CI=0.75-0.99, P=0.030), while no association was observed for mortality (OR=0.89, 05% CI=0.78-1.00, P=0.128; Table 10). miR-200c expression did not show any association with other clinicopathological characteristics. No association with treatment response was found for the other miR-200 family members.

Table 10. miR-200c expression and response to treatment.

	Nº samples	miR200c Mean (ru)	miR200c 95%CI (ru)	P value [a] (univariate)	P value [b] (multivariate)
Response					
CR	37	9.1	(6.8-11.3)	**0.0027[d]**	**0.037**
No CR (PR, SD, PD[c])	17	4.8	(3.1- 6.5)		
Pathological response					
CR	14	8.0	(5.6-10.3)	**0.0450**	0.094
No CR	10	4.6	(2.0- 7.1)		
Recurrence					
No	8	11.8	(3.1-20.6)	0.2429[d]	0.030
Yes	45	7.0	(5.5- 8.5)		
Deceased					
No	19	9.3	(5.4-13.1)	0.2487[d]	0.128
Yes	35	6.9	(5.0- 8.7)		

[a] Univariate analysis using unpaired t-test.
[b] Multivariate analysis using logistic regression with debulking status, tumor grade and FIGO stage as covariables.
[c] Complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD).
[d] Welch correction was applied when the standard deviations differed significantly between groups.

A high tumoral class III protein expression has been associated with poor clinical outcome in several cancers. However, in our samples set, we did not find a statistically significant association between high class III content and the clinical response and survival of the patients. No association was found neither between class II β-tubulin expression and clinical response.

The expression of the miR-200 family did not present any association with other clinical or tumoral characteristics. As for disease outcome, only miR-429 expression showed a statistically significant association with recurrence-free and overall survival of the patients in a univariant analysis (Figure 16). After multivariable analysis adjusting for relevant clinicopathological variables (debulking status, tumor stage and histological grade) a tendency was observed for miR-429 with recurrence-free survival (HR=0,477, 95%CI=0.21-1.09, p=0.080). Similarly, miR-200c and miR-141 also showed a similar trend (Table 11). The association with overall survival was lost for miR-429 in the multivariant analysis and none of the other miRNAs showed any tendency.

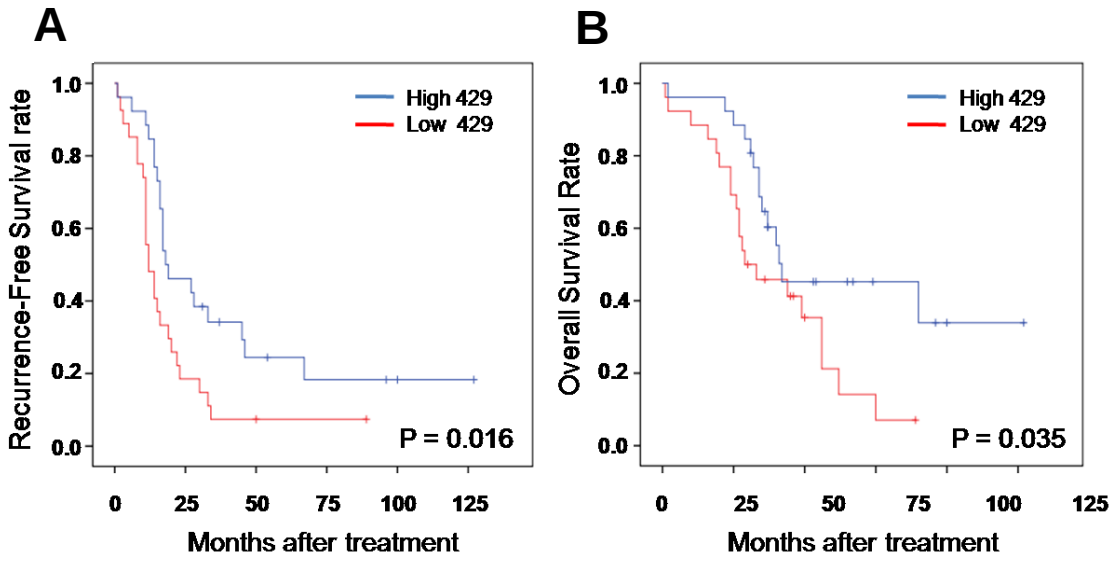

Figure 16. Mir-429 is associated with better recurrence-free and overall survival. Kaplan-Meier survival analysis evaluating the effect of miR-429 expression on disease outcome for A) recurrence-free and B) overall survival.

Table 11. miR-200 family expression and progression-free survival.

	HR (95% CI) [a]	P value
miR-200c	0.45 (0.20-1.00)	0.051
miR-200b	0.74 (0.34-1.61)	0.450
miR-200a	0.83 (0.39-1.75)	0.612
miR-141	0.43 (0.180-1.02)	0.054
miR-429	0.48 (0.21-1.09)	0.080

[a]Hazard ratio for relapse calculated using multivariate Cox regression analysis with debulking status, tumor grade and FIGO stage as covariables.

Results, part III

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

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

Since there are previous evidences suggesting that genetic alterations in paclitaxel pharmacokinetic pathway could underlie the interindividual differences in the neurotoxicity exhibited by the patients treated with this drug, we followed a candidate-gene approach aiming at the identification of polymorphisms associated with paclitaxel neurotoxicity.

3.1. Selection of SNPs potentially influencing paclitaxel neurotoxicity and observed allelic frequencies in Spanish patients

Six genes involved in paclitaxel metabolism and transport (*CYP3A4*, *CYP3A5*, *CYP2C8*, *SLCO1B1, SLCO1B3* and *ABCB1*; see Figure 4 and Table 12) were selected for the study, and thirteen SNPs were included based on functionality and allele frequency in Caucasians. All the SNPs selected for the study had reported minor allele frequencies greater than 5% in white Europeans (Table 12).

For *CYP2C8* we selected four SNPs (rs11572080, rs1058930, rs1113129 and rs79092356). Several studies have demonstrated that *CYP2C8*3* (R139K; K399R) exhibits an altered activity (Aquilante et al. 2008; Dai et al. 2001; Kirchheiner et al. 2008; Niemi et al. 2005; Niemi et al. 2003; Soyama et al. 2001). *CYP2C8*4* (I264M) has not been associated to an altered enzyme activity, (Niemi et al. 2005) but it encodes a common variant protein which may not have been studied thoroughly. *CYP2C8* haplotypes B and C, (represented by rs7909236 (*CYP2C8*1B*) and rs1113129 (Rodriguez-Antona et al. 2008)), were recently shown to confer an increased and reduced activity, respectively. (Rodriguez-Antona et al. 2008)

For *CYP3A4* there are no common coding polymorphisms and the only variant repeatedly studied is the promoter *CYP3A4*1B* allele, although its effect on enzyme activity remains unclear (Rodriguez-Antona et al. 2005). In contrast, *CYP3A5* is very polymorphic, and its activity in Caucasians is determined by *CYP3A5*3* allele through alternative splicing (Kuehl et al. 2001).

For the uptake transporters OATP1B1 and OATP1B3 (encoded by *SLCO1B1* and *SLCO1B3* genes) we selected missense polymorphisms with reported functional consequences. (Smith et al. 2007) Finally, for *ABCB1* we selected the three most studied variants (1236C>T, 2677G>T, 3435C>T) (Leschziner et al. 2007) and a

missense (N21D) polymorphism described in public databases but without previous results.

Upon genotyping of the samples, the call rates were very high for the 13 selected polymorphisms (genotypes were successfully called for between 116 and 118, of the 118 samples). The minor allele frequencies of the polymorphisms (Table 12) were similar to those previously described for Caucasians (Kimchi-Sarfaty, et al. 2007; Kuehl et al. 2001; Niemi et al. 2005; Rodriguez-Antona et al. 2008; Smith et al. 2007) and all the SNPs were in Hardy-Weinberg equilibrium. The frequency of the different alleles is shown in Table 12. The two *SLCO1B3* SNPs analyzed (rs4149117 and rs7311358) were in complete linkage disequilibrium (r^2=1.0).

3.2. Paclitaxel neurotoxicity is influenced by treatment schedule and age

When we compared the neurotoxicity data with demographic and treatment related data, as expected, we found that the weekly, more intense, 80-90 mg/m^2 paclitaxel schedule was more neurotoxic than the 150-175 mg/m^2 scheme administered every 21 days (P=0.043) (Figure 17A). In addition, patients younger than 50 years showed a significantly higher neurotoxicity than those older than 50 years (P=0.019) (Figure 17B). The estimated hazard ratio (HR) for these two factors were 1.70 (95% CI=1.01-2.87) and 2.12 (95% CI=1.12-4.02), respectively. No evidence of association with neurotoxicity was found for other demographic (e.g. gender, BSA) and clinical factors (e.g. type of tumor, previous neurotoxicity treatments and comorbidity factors).

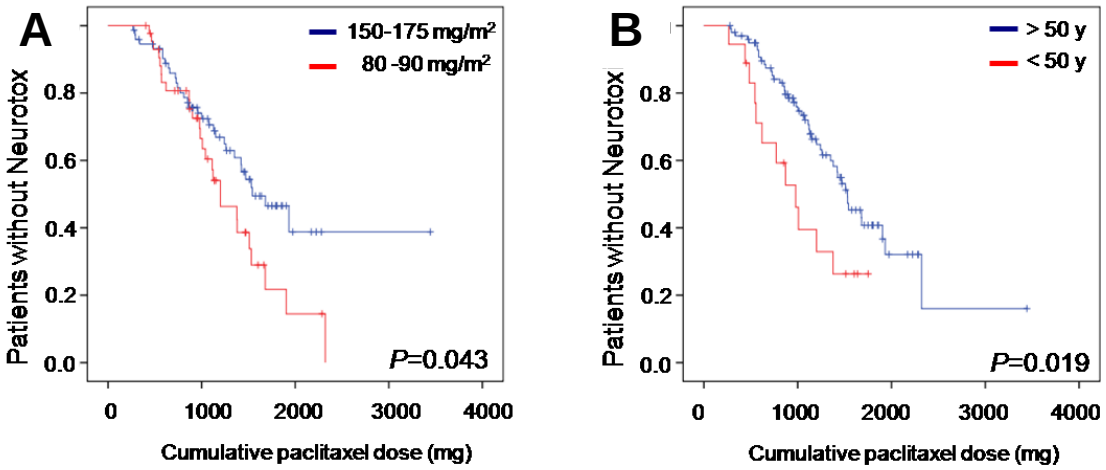

Figure 17. Paclitaxel neurotoxicity is influenced by treatment schedule and age. Kaplan-Meier estimates of paclitaxel neurotoxicity modelling the cumulative dose of paclitaxel up to the development of grade 2 neurotoxicity. A) Paclitaxel administered in 80-90 mg/m2 schedules was more neurotoxic than 150/175 mg/m^2; p(log-rank)=0.043; HR=1.70, 95% CI=1.01-2.87. B) Patients younger than 50 years of age showed a significantly higher neurotoxicity than those older than 50 years; p(log-rank)=0.019; HR=2.12, 95% CI=1.12-4.02.

*3.3. CYP2C8 Haplotype C and CYP3A5*3 are associated with protection while CYP2C8*3 is associated with increased risk of paclitaxel neurotoxicity*

Three polymorphisms showed statistically significant evidence of association with paclitaxel neurotoxicity risk: *CYP2C8*3* (Figure 18A), CYP2C8 Haplotype C (Figure 18B) and *CYP3A5*3* (Figure 18C) (P=0.049, 0.049 and 0.010, respectively). Multivariable Cox regression adjusting for treatment schedule and age, gave an estimated HR of 1.72 (95%CI=1.05-2.82; P=0.032) for *CYP2C8*3*; 0.55 (95%CI=0.34-0.89; P=0.014) for *CYP2C8* Haplotype C and 0.51 (95%CI=0.30-0.86; P=0.012) for *CYP3A5*3* (see Table 12).

Table 12. Allele frequency of the genetic variants and their associated risk with paclitaxel neurotoxicity.

	dbSNP	Common allele name	Amino acid change	SNP effect	Allele Freq [a]	HR (95% CI)[b]	P value Cox [b]	HR (95% CI)[c]	P value Cox [c]
CYP2C8	**rs11572080,G>A**	***CYP2C8*3***	**R139K;(K399R)**	**Variant protein**	**0.18**	**1.54 (0.96-2.47)**	**0.072**	**1.72 (1.05-2.82)**	**0.032**
CYP2C8	rs1058930, C>G	*CYP2C8*4*	I264M	Variant protein	0.07	0.68 (0.29-1.57)	0.361	0.53 (0.22-1.26)	0.150
CYP2C8	**rs1113129, G>C**	***CYP2C8-HapC***	**-**	**Haplotype low act**	**0.25**	**0.59 (0.36-0.94)**	**0.026**	**0.55 (0.34-0.89)**	**0.014**
CYP2C8	rs7909236, G>T	*CYP2C8*1B*	-	Variant promoter	0.15	0.86 (0.50-1.49)	0.593	0.86 (0.49-1.52)	0.611
CYP3A4	rs2740574, A>G	*CYP3A4*1B/V*	-	Variant Promoter	0.06	1.62 (0.76-3.43)	0.209	2.13 (0.98-4.66)	0.057
CYP3A5	**rs776746, A>G**	***CYP3A5*3***	**-**	**Splicing defect**	**0.92**	**0.55 (0.33-0.94)**	**0.027**	**0.51 (0.30-0.86)**	**0.012**
ABCB1	rs2032582, G>T	2677	A893S	Variant protein	0.35	1.22 (0.84-1.78)	0.295	1.23 (0.84-1.79)	0.286
ABCB1	rs1128503, C>T	1236	G412G	Unknown	0.40	1.01 (0.70-1.46)	0.970	0.94 (0.65-1.35)	0.727
ABCB1	rs1045642, C>T	3435	I1145I	Unknown	0.41	0.74 (0.50-1.11)	0.142	0.71 (0.47-1.06)	0.093
ABCB1	rs9282564, A>G	-	N21D	Variant protein	0.05	0.62 (0.26-1.46)	0.272	0.43 (0.17-1.04)	0.061
SLCO1B1	rs4149056, T>C	-	V174A	Variant protein	0.15	0.91 (0.52-1.57)	0.726	0.82 (0.47-1.45)	0.504
SLCO1B3	rs4149117[d], G>T	-	S112A	Variant protein	0.17	1.15 (0.68-1.94)	0.596	1.18 (0.69-2.00)	0.549
SLCO1B3	rs7311358[d], A>G	-	M233I	Variant protein	0.17	1.15 (0.68-1.94)	0.596	1.18 (0.69-2.00)	0.549
Model high act. variants	**rs11572080A rs1113129G rs776746G**					**1.51 (1.17-1.94)**	**0.001**	**1.64 (1.26-2.14)**	**0.0003**

[a] Variant allele frequency in Spanish cancer patients.
[b] Hazard ratios, confidence intervals and P values calculated by Cox regression.
[c] Hazard ratios, confidence intervals and P values estimated by multivariable Cox regression, including treatment schedule (80-90 mg/m^2 *versus* 150-175mg/m^2) and age as covariates.
[d] These two SNPs were in complete linkage disequilibrium (r^2=1.0).

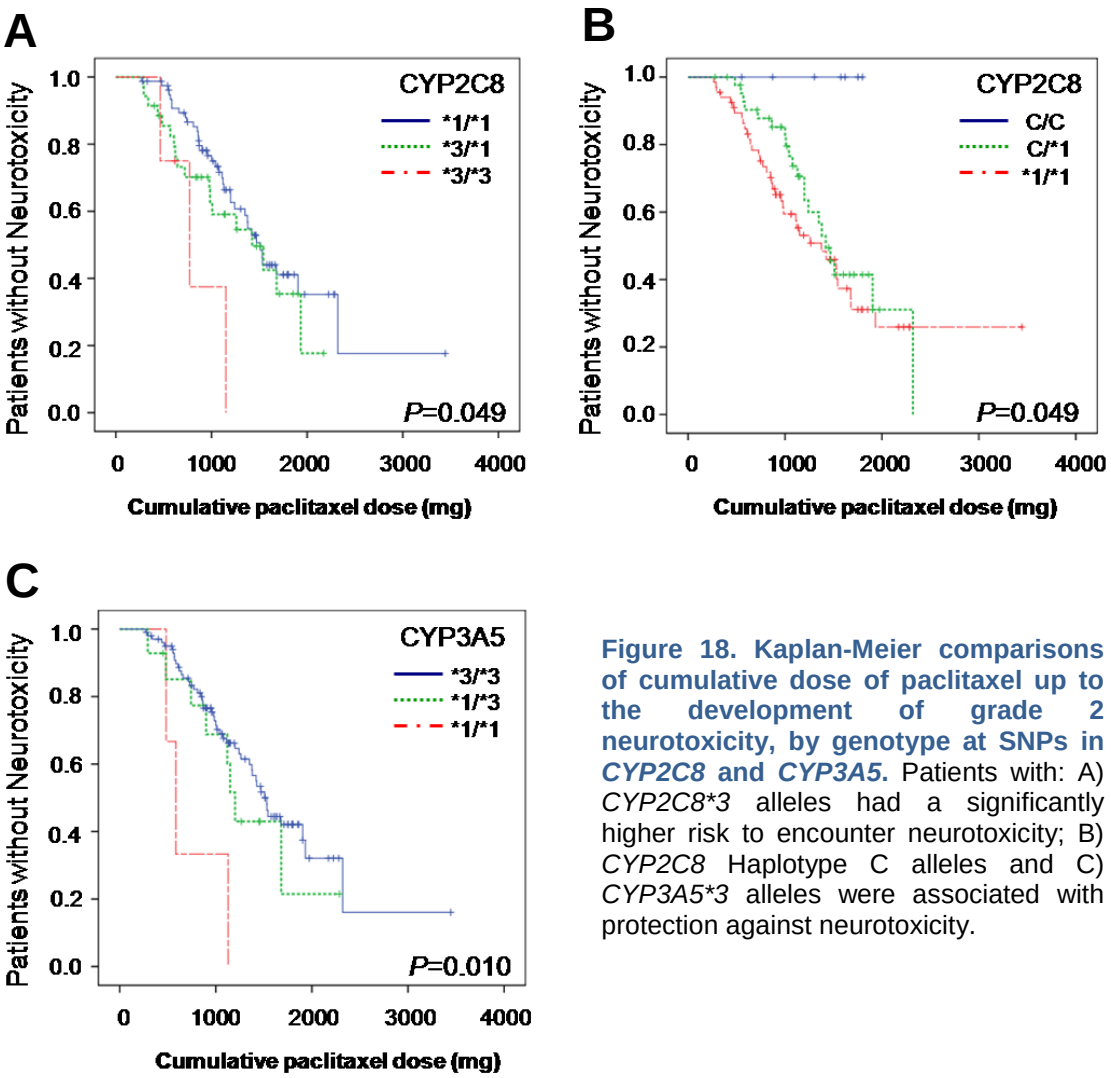

Figure 18. Kaplan-Meier comparisons of cumulative dose of paclitaxel up to the development of grade 2 neurotoxicity, by genotype at SNPs in *CYP2C8* and *CYP3A5*. Patients with: A) *CYP2C8*3* alleles had a significantly higher risk to encounter neurotoxicity; B) *CYP2C8* Haplotype C alleles and C) *CYP3A5*3* alleles were associated with protection against neurotoxicity.

No evidence of association was observed for the uptake transporters *SLCO1B1* and *SLCO1B3* or the *ABCB1* efflux pump (Table 12). The statistical analyses were repeated after normalising the cumulative paclitaxel doses by BSA, without any substantive changes in the results obtained.

3.4. CYP-based single prediction model to predict paclitaxel neurotoxicity

Initial *in vitro* studies for *CYP2C8*3* suggested a lower activity, but studies in healthy volunteers have demonstrated an increased metabolizing capacity leading to higher production of hydroxylated paclitaxel metabolites. Interestingly, we found that *CYP2C8*3* was associated with higher neurotoxicity risk. While the SNPs associated with protection (*CYP2C8* haplotype C and *CYP3A5*3*) confer a reduced

activity. All the three SNPs act in an independent manner, the two SNPs in *CYP2C8* are not in linkage disequilibrium (r2=0.005) and *CYP3A5*3* is located on a different chromosome. Thus we constructed a model including these three polymorphisms acting in a log-additive, per-allele fashion and estimated the HR per high-paclitaxel-metabolizing allele to be 1.64 (95%CI=1.26-2.14, P=0.0003) after correcting by treatment schedule and age (Table 12 and Figure 19).

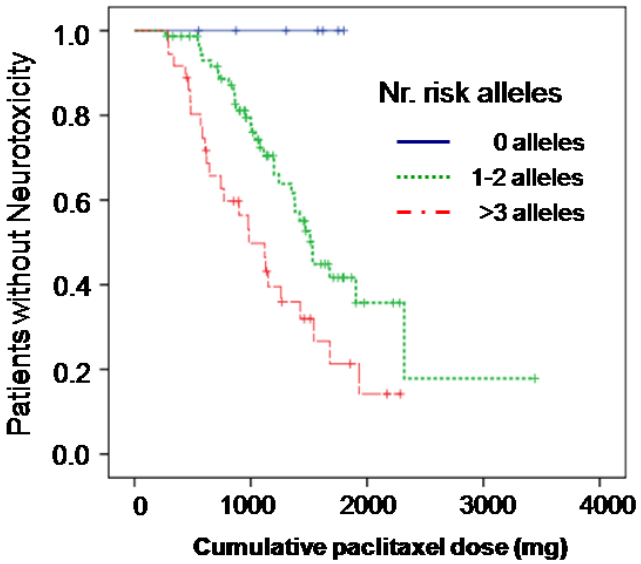

Figure 19. Proposed model for paclitaxel neurotoxicity risk including the 3 identified polymorphisms acting in a log-additive, per-allele fashion. Patients were classified in three groups according to the number of risk alleles (0 to 5). The Kaplan-Meier analysis shows the comparisons of cumulative dose of paclitaxel up to the development of grade 2 neurotoxicity by number of the risk alleles: rs11572080A, rs1113129G and rs776746G

In this manner, taking into account the total nº of high-neurotoxicity risk alleles, individuals can be classified in seven groups depending on the number of risk alleles (0-6). While inheriting five or six risk alleles is infrequent in Caucasians, carrying zero to four alleles is relatively common (frequency 3-45%).

RESULTADOS

1. Bloque I: *CYP3A5* se expresa en próstata a altos niveles, pero está ausente en el tejido tumoral

El primer estudio de esta Tesis doctoral estuvo centrado en la determinación de la expresión de los cuatro enzimas CYP3A en tejidos normales y tumorales de próstata, y en la identificación de los mecanismos que controlan la expresión de estos genes. Descubrimos que de los cuatro genes *CYP3A* humanos, sólo el *CYP3A5* se expresaba en niveles relevantes (mRNA y proteína) en próstata, en concreto en las células basolaterales. La expresión prostática del CYP3A5 presentaba una gran variabilidad interindividual que se explicaba por el polimorfismo *CYP3A5*3*. Con respecto a los tejidos tumorales descubrimos que la expresión del CYP3A5 desaparecía.

2. Bloque II: *La familia miR-200 regula la expresión de la β-tubulina III y se asocia con la respuesta al tratamiento y supervivencia libre de recaída de pacientes con cáncer de ovario tratados con paclitaxel-carboplatino*

El segundo proyecto se centró en el estudio de la diana terapéutica de los taxanos, la β-tubulina. Mediante la caracterización de la expresión proteica de los isotipos I, II y III de la β-tubulina y de un estudio de expresión de microRNAs mediante RT-PCR cuantitativa, demostramos que la familia miR-200 regulaba la expresión del isotipo III. Además, en pacientes con cáncer de ovario avanzado los niveles de expresión del miR-200c se asociaron de forma estadísticamente significativa con la respuesta (P=0.0027 con t-test; HR=0.23, 95%CI=0.06-0.84, P=0.026 en análisis multivariante). También se encontró una asociación con la respuesta patólogica en análisis univariante (P=0.045), aunque la asociación no llegó a ser significante en un análisis multivariante (HR=0.19, 95%CI=0.03-1.31, P=0.092). Por otra parte, los niveles de expresión del miR-429, miR-141 y miR-200c mostraron una tendencia de asociación con la recaida riesgos relativos HR=0,477 (95%CI=0.21-1.09, P=0.080), HR=0,427 (95%CI=0.18-1.02, P=0.054) y HR=0,447 (95%CI=0.20-1.00, P=0.051), respectivamente.

3. Bloque III: *Polimorfismos en los Citocromos P450 2C8 y 3A5 se asocian con la neurotoxidad del paclitaxel*

En el tercer proyecto nos centramos en la identificación de marcadores genéticos predictores de neurotoxicidad por paclitaxel utilizando una estrategia de genes candidatos. Para ello se genotipó una selección de polimorfismos en los genes implicados en la metabolización (CYP2C8, CYP3A4 y CYP3A5) y transporte (SLCO1B1, SLCO1B3 y P-glicoproteina) del paclitaxel en 118 pacientes tratados

con este fármaco y de los que se disponía de datos de toxicidad. Descubrimos que tres polimorfismos (uno en el gen *CYP3A5* y dos en el *CYP2C8*) se asociaban de forma estadísticamente significativa con la neurotoxicidad del paclitaxel: el *CYP2C8* Haplotipo C y el *CYP3A5*3* confirieron protección frente al riesgo de desarrollar neurotoxicidad (HR(por alelo)=0.55, 95%CI=0.34-0.89, P=0.014 y HR(por alelo)=0.51, 95%CI=0.30-0.86, P=0.012, respectivamente) mientras que el *CYP2C8*3* confirió un mayor riesgo (HR(por alelo)=1.72, 95%CI=1.05-2.82, P=0.032). Finalmente se creó un modelo que incluía los tres polimorfismos actuando en un modo aditivo, dando un riesgo relativo por cada alelo de alta-metabolización de paclitaxel de 1.64 (95%CI=1.26-2.14, P=0.0003). Los resultados para la P-glicoproteína no fueron concluyentes, y no se observó ninguna asociación para los otros genes estudiados.

DISCUSSION

Discussion, part I

Cytochrome P450 3A5 is highly expressed in normal prostate cells but absent in prostate cancer

1. CYP3A5 expression in prostate: potential relevance for prostate cancer risk and for docetaxel efficacy in prostate cancer patients

Prostate cancer (PC) is the second leading cause of cancer death in men, and both genetic and environmental factors have been shown to be important for the development of this disease (Novelli, et al. 2004). More than 95% of the prostate tumors derive from luminal cells, which grow in an androgen-dependent manner through androgen receptor activation. Testosterone is essential for prostate cancer development (Feldman and Feldman 2001) and genetic changes affecting the expression/ activity of the enzymes metabolizing androgens can influence prostate cancer progression (Makridakis, et al. 1997; Makridakis, et al. 1999; Park, et al. 2006; Rebbeck, et al. 2008; Vaarala, et al. 2008). For this reason, several studies have focused on *CYP3A* polymorphisms, the rationale being that, in addition to the prominent role of CYP3A enzymes in the metabolism of over 50% of all clinical drugs (Li, et al. 1995; Rodriguez-Antona and Ingelman-Sundberg 2006; Thummel and Wilkinson 1998), CYP3A enzymes also metabolize testosterone and dehydroepiandrosterone (DHEA) to less active hydroxy-metabolites (Kamdem, et al. 2004; Miller, et al. 2004; Ohmori, et al. 1998). Thus, an alteration in the CYP3A prostatic activity could change the local testosterone levels and the tissue-specific androgen effects, which could alter prostate cells growth and lead to cancer development.

Prostate cancer is usually treated with hormonal therapy; however, most cases evolve to a hormonal-resistance stage. At this point, docetaxel chemotherapy offers both symptomatic and survival benefits in men with metastatic hormone-refractory prostate. However, only 48% to 50% of patients respond to docetaxel treatment, thus in approximately half of the cases the therapy is not effective. In addition, the median increase in overall survival is only few months, as docetaxel therapy ultimately fails to control the progression of the cancer (Petrylak, et al. 2004; Tannock, et al. 2004). CYP3A enzymes hydroxylate docetaxel to less active metabolites (Vaclavikova, et al. 2004). Thus *CYP3A* polymorphisms might modify the efficacy of docetaxel treatment by altering its inactivation in the prostate cancer cells.

Despite to the relevance that CYP3A enzymes could have in the prostate, when this thesis started the prostatic expression of the CYP3A enzymes was mainly unknown.

1.1 CYP3A5 is expressed at high levels in the basolateral cells of normal prostate tissue and its expression is influenced by CYP3A5*3 polymorphism

The CYP3A subfamily comprises four members: CYP3A4, CYP3A5, CYP3A7 and CYP3A43, which have similar substrate specificities but are expressed at different levels and with different expression patterns: CYP3A7 is mainly expressed at fetal stages in the liver, CYP3A4 and CYP3A5 are mainly expressed in the adult liver and intestines and CYP3A43 has been suggested to be expressed at very low levels in different tissues (Lin, et al. 2002). Contradictory results have been reported with respect to the expression of the CYP3A enzymes in prostate tissue (Di Paolo, et al. 2005; Finnstrom, et al. 2001; Fujimura, et al. 2009; Koch, et al. 2002; Moilanen, et al. 2007; Murray, et al. 1995; Stamey, et al. 2001; Zhang, et al. 2006). In this work we showed that of the four human CYP3A enzymes only CYP3A5 had a relevant expression in prostate, with mRNA contents similar to those in liver (Figure 7). Specifically, when comparing the CYP3A5 mRNA content of liver samples with prostate samples, the prostate samples showed about 20% of the hepatic levels (Figure 8).

Among the prostate samples included in this study, there was a large CYP3A5 mRNA and protein variation, mainly caused by *CYP3A5*3* polymorphism, which introduces an alternative splicing site that in the liver decreases the amount of full-coding transcript and ultimately causes low CYP3A5 protein expression (Kuehl, et al. 2001). In this work, *CYP3A5*1/*3* prostate samples had 13-fold higher amounts of correctly spliced mRNA than *CYP3A5*3/*3* samples and, although the number of samples analyzed was small and the correlation with mRNA levels was not strong, the highest CYP3A5 protein content also corresponded to CYP3A5*1/*3* prostate samples (Figure 8B results). Moilanen *et al.* detected CYP3A5 protein in the prostate, but surprisingly *CYP3A5*3/*3* samples showed similar or even higher CYP3A5 protein content than *CYP3A5*1/*3* samples, and by IHC they found CYP3A5 protein both in luminal and basolateral cells and both in non-tumoral and tumoral prostate tissue (Moilanen et al. 2007). In contrast, in this work we found that CYP3A5 was exclusively expressed in the basolateral cells of the non-tumoral tissue and absent in the tumoral tissue (Figure 9). Our data was fully supported by the quantitative RT-PCR analysis we performed in the matched non-tumoral/ tumoral samples (Table 8). The differences between this and other studies can be explained by the use of antibodies with low specificity (Moilanen et al. 2007) and by assessing

mRNA by non-quantitative PCR methods (Finnstrom, et al. 2001; Stamey, et al. 2001). Thus, in this study we unequivocally establish for the first time a relevant expression of the CYP3A enzymes in prostate tissue, specifically of CYP3A5.

1.2. CYP3A5 and androgen metabolism

Because CYP3A5 protein is exclusively localized in the basolateral cells, and these represent 2% of the total prostate cells (Liu, et al. 1997) about 7.5 pmol of CYP3A5/ mg microsomal basolateral protein can be estimated, which is similar to the 37 pmol found in *CYP3A5*1/*3* livers (Liu et al. 1997; Westlind-Johnsson, et al. 2003). Similarly, if we take into account that only the basolateral cells contribute to CYP3A5 mRNA expression, the CYP3A5 mRNA levels in the prostate basal cells would be 10-times higher than in the liver cells. The difference between the prostatic and hepatic CYP3A5 mRNA and protein contents could indicate a tissue-specific *CYP3A5* post-transcriptional regulation. In any case, the high CYP3A5 prostatic expression suggests that CYP3A5 must play a relevant function in the prostate and, since the prostate is not a tissue relevant for drug metabolism, this function must be related to the metabolism of prostatic endogenous CYP3A5 substrates, such as androgens (Miller et al. 2004; Ohmori et al. 1998). In other tissues, CYP3A5 has also been shown to play an important endogenous function, and *CYP3A5*3* has been shown to influence the systolic blood and pulse pressure, presumably by altering CYP3A5-mediated glucocorticoid metabolism (Kreutz, et al. 2005). Thus, by oxidation of testosterone and DHEA in the basolateral prostate cells, CYP3A5 could control their entrance into the luminal cells which grow in a hormone-dependent manner (Masai, et al. 1990). In fact, androgens up-regulate CYP3A5 expression in human prostate, suggesting an autoregulatory loop to control testosterone exposure (Moilanen et al. 2007). Consequently, polymorphism affecting CYP3A5 activity could alter the oxidation of androgens in the basolateral cells and ultimately luminal prostate cell growth, function and prostate cancer development (Parnes, et al. 2005; Zhenhua, et al. 2005).

1.3. The role of CYP3A5 polymorphisms in prostate cancer development

In line with this idea, several publications have associated *CYP3A* SNPs with prostate cancer risk and aggressiveness (Bangsi, et al. 2006; Loukola, et al. 2004; Paris, et al. 1999; Plummer, et al. 2003; Rebbeck, et al. 1998; Vaarala et al. 2008;

Zhenhua et al. 2005). However, most studies have focused on *CYP3A4*1B,* which does not result in major changes in CYP3A4 expression (Rodriguez-Antona, et al. 2005), and which, is not expressed in the prostate (Figure 7). The *CYP3A* locus shows a high degree of linkage disequilibrium (Kuehl et al. 2001; Lee, et al. 2003), and approximately 80% of Caucasians carrying *CYP3A4*1B* are simultaneously *CYP3A5*1* (Wojnowski, et al. 2002). In Asians, in which *CYP3A4*1B* is absent and, thus, is not a confounding factor, *CYP3A5*1/*1* men had a 0.23-fold lower risk of developing a low-grade prostate cancer and a 0.31-fold lower risk of developing localized prostate cancer than *CYP3A5*3/*3* men (Zhenhua et al. 2005). In Africans, in addition to *CYP3A5*3,* there are two other common functional *CYP3A5* polymorphisms which have not been taken into account in any of the association studies carried out so far: *CYP3A5*6*, which creates an alternative splicing site similarly to *CYP3A5*3*, and *CYP3A5*7* which has a single nucleotide insertion causing a frameshift and early stop codon (with 13% and 10% allele frequency, respectively) (Hustert, et al. 2001; Kuehl et al. 2001; Lee et al. 2003). Therefore, the association studies in Africans can only be complete when all three *CYP3A5* defective alleles are considered together and compared with the wild type *CYP3A5*1* allele. Undoubtedly, further studies are needed to confirm the association of *CYP3A5* functional SNPs with prostate cancer.

1.4. The efficacy of docetaxel in prostate cancer treatment is unlikely to be modified by CYP3A5 polymorphisms

The lack of CYP3A5 expression in the tumoral tissue indicates that the efficacy of docetaxel treatment is unlikely to depend on the *CYP3A5* polymorphisms. Several genes have been associated with docetaxel resistance, Bcl-2 (Tolcher, et al. 2005) and Stat1 (Patterson, et al. 2006), for instance. However, drugs targeting these pathways have either not been tested, or they have failed to improve docetaxel efficacy in clinical trials (Petrylak et al. 2004; Tannock et al. 2004; Tolcher et al. 2005). Thus, novel biomarkers associated with docetaxel resistance still need to be identified in order to enhance survival of prostate cancer patients.

In conclusion, we have shown that only *CYP3A5* out of the four *CYP3A* genes is expressed at high levels in the non-tumoral prostate tissue, specifically in the basolateral cells and that this expression does not occur in the tumors. This data reveals an important endogenous role of CYP3A5 in the prostate and association

studies between prostate cancer and *CYP3A* polymorphisms indicate that this function must be related to the metabolism of intra-prostatic androgens and regulation of luminal cell growth. Furthermore, this data suggests that future prostate cancer association studies on *CYP3A* genes should focus on *CYP3A5* functional polymorphisms such as *CYP3A5*3, *6* and **7* which could be directly associated with prostate cancer risk and aggressiveness. On the other hand, the lack of CYP3A5 expression in the tumoral prostate tissue suggests that this enzyme cannot directly affect the efficacy of docetaxel treatment in the cancer cells.

Discussion, part II

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

2. miR-200 family regulates class III β-tubulin expression and predicts treatment outcome in ovarian cancer

Ovarian cancer is one of the leading causes of cancer deaths. Most ovarian cancers are detected at advanced stages and, despite advances in cytotoxic therapies, 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 identification of biomarkers predicting treatment outcome. This study is focused on the new field of microRNAs because of their potential to provide novel drug response markers (Adam, et al. 2009; Li, et al. 2009; Yang, et al. 2008a; Yang, et al. 2008b) and establishes miR-200 family as an important factor for paclitaxel-carboplatin response in ovarian cancer.

2.1. miR-200 expression determines class III β-tubulin content in ovarian tumors

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 tumour progression (Gregory, et al. 2008). Interestingly, by *in silico* tools we found that miR-200c/b/429 had putative binding sites in the 3' UTR of the β-tubulin isotypes I, II and III (Figure 11). Since β-tubulin is the therapeutic target of paclitaxel, we hypothesized that these miRNAs might influence the response of ovarian cancer to paclitaxel-based treatments through down-regulation of these three isotypes in tumoral cells. From the 5 miR-200 family members, the seed sequence of miR-141 and miR-200a differ by only one nucleotide with that of miR-200b, miR-200c and miR429 and, although target prediction algorithms assume significant differences in the genes targeted by miR-200b/c/429 and miR-200a/141, there are evidences indicating a much higher 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 in order to enhance the efficiency of genetic regulation. Thus, we measured the expression of the five miR-200 family members in 72 epithelial ovarian cancer samples, and observed, in agreement with previous reports, that the expression of these miRNAs correlated (Table 9) and that miR-200c, miR-200b and miR-200a had higher expression than miR-141 and miR-429 (Hu, et al. 2009; Park et al. 2008).

We analyzed by IHC the expression of class I, II and III β-tubulins, finding class I homogenously expressed at high levels in all samples, class II present in 31% and III in 52% of the tumors, although a high expression (>75% of positive cells) was observed in only 9% and 11% of the tumours, respectively. These results are in agreement with those reported in a previous ovarian series (Ohishi, et al. 2007). In our series, the different tumoral histologies did not seem to influence the expression of the isotypes, although most of the cases were serous cystoadenocarcinomas. Umezu *et al.* using cases representing the different histologies, reported higher β-tubulin III protein levels in clear cell and mucinous types compared to serous and endometrioid tumors (Umezu, et al. 2008). In other tumor types, expression of isotype III has been reported in 40, 36, 84% of head and neck carcinoma (Koh, et al. 2009), gastric cancer (Urano, et al. 2006) and breast cancer (Paradiso, et al. 2005) samples, respectively. For isotype II, lack of expression seems to be associated with advanced stage and short progression free survival in ovarian cancer (Ohishi et al. 2007). Interestingly, we found a mutual exclusivity for class II and III β-tubulin expression (Figure 13). This data suggests that, although the three isotypes had predicted binding sites for miR-200b/c/429, different regulation mechanisms, still unknown, were determining the final protein expression levels of the tumors.

Tumoral over-expression of class III β-tubulin has been associated with poor prognosis in a variety of cancer types (Koh et al. 2009; Seve and Dumontet 2008; Seve, et al. 2005) including ovarian carcinomas (Ferrandina, et al. 2006). These findings seem to reflect, at least in part, an increased resistance of class III microtubules to the effect of microtubule-binding drugs (Cochrane, et al. 2009). In our series, we were not able to detect a significant association between β-tubulin III expression and treatment response, which could be due to the small number of samples with high class III expression. Previously, high class III protein expression has been associated with lack of response to taxanes in several studies including breast (Hasegawa, et al. 2003; Paradiso et al. 2005), lung (Rosell, et al. 2003; Seve et al. 2005) and ovarian (Umezu et al. 2008) tumors. In contrast, one study reported high β-tubulin III associated to a better overall and progression-free survival in clear cell ovarian cancer patients, although they did not provide information on treatment response (Aoki, et al. 2009). This data suggests that histological differences may play an important role in the association of class III β-tubulin expression and survival.

When we studied the relation of the miR-200 family with the protein expression of the β-tubulin isotypes, we found that low levels of miR-200 were associated with high levels of class III protein, demonstrating for the first time in tumours that β-tubulin III expression is determined by miR-200 family (Figure 15). A recent study, by Cochrane *et al*. showing that the reinstatement of miR-200c in cell lines decreases class III β-tubulin expression, supports this finding (Cochrane et al. 2009). Although low expression of miR-200 is necessary for the over-expression of isotype III, other mechanisms must also be involved as there are tumours with low levels of both, miR-200 and isotype III. We did not find an association between miR-200 and class I and II protein expression, which is compatible with the IHC findings and supports the notion that the regulation of these isotypes is different from that of class III. Thus, the predicted binding sites for miR-200b/c/429 in class I and II are either non-functional or alternative mechanisms determine their final protein expression. For class II the predicted binding site was only conserved among mammals, suggesting the first possibility. However, for class I the predicted binding site was broadly conserved among vertebrates, similarly to isotype III.

2.2. The patients' response to taxane-based treatment and progression-free survival are associated with miR-200 expression

As to a possible role for miR-200 on paclitaxel sensitivity, we found a significant association between miR-200c expression and treatment response: women with complete response had tumours with significantly higher miR-200c levels than those lacking complete response (HR=0.70, 95% CI=0.50-0.98, P=0.037). Also, higher expression of miR-200c was associated with protection against recurrence (OR=0.86 95% CI=0.75-0.99 P=0.030). This data suggests that low miR-200c expression leads to increased β-tubulin III expression and, thus, enhanced resistance to paclitaxel-based therapies. Previous *in vitro* studies support that miR-200c increases sensitivity to microtubule-targeting agents (Cochrane et al. 2009).

Concerning prognosis, we found that in our series that low tumoral miR-429 was associated with poor progression-free and overall survival (Figure 16). Adjustment with relevant clinicopathologic variables revealed a tendency for progression-free survival for miR-429, miR-200c and miR-141 (Table 11), while the association with overall survival was lost. No trends were observed for the remaining microRNAs of the miR-200 family. In a recent study, low miR-200a expression was

associated with poor recurrence-free and overall survival in ovarian cancer patients (Hu et al. 2009). On the other hand, Nam *et al.* found that a high expression of miR-200b/c/429 was associated with poor survival (Nam, et al. 2008). The discrepant results found by Nam *et al.* could be caused by the small number of samples included in the study (20 serous ovarian tumour samples).

Low tumoral expression of the miR-200 family has been associated with tumor progression and metastasis (Baffa, et al. 2009; Gregory et al. 2008; Park et al. 2008), which could lead to a lower overall survival, independently of the treatment response. Our results suggest a possible role of miR-200 family as a predictive factor for paclitaxel-based response, especially miR-200c, and as a prognostic factor in ovarian carcinoma (Figure 20). Because all miR-200 family members share similar targets, but enclose differences in the recognition site, we propose that specific members of the family will be more important for prognosis and others for treatment response. In addition, the relative expression levels in the tumor cells could play a part in the final regulation of the target genes. Thus, low tumoral miR-200 family expression could act two-fold: decreasing response to microtubule-binding drugs and increasing metastasis through increased epithelial to mesenchymal transition.

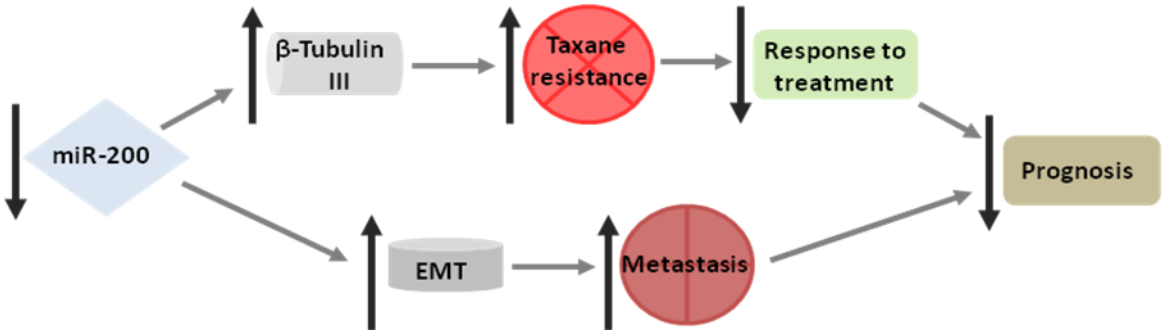

Figure 20. Proposed function of miR-200 family as a prognostic factor and a marker of treatment failure in ovarian carcinoma. Low miR-200 expression allows higher B-tubulin III expression causing increased taxane resistance, which leads to lack of response and poor prognosis, while on the other hand low miR-200 also permits EMT and brings about metastasis thus leading to poor prognosis. EMT, Epithelial to mesenchymal transition

Altogether, we have demonstrated that miR-200 down-regulates class III β-tubulin expression in ovarian tumours. 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 tumour cells.

Discussion, part III

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

3. Polymorphisms in *CYP2C8* and *CYP3A5* are associated with paclitaxel neurotoxicity

This pharmacogenetic study was designed to identify genetic variants influencing paclitaxel induced peripheral neurotoxicity, which is the major side-effect and the dose-limiting toxicity of paclitaxel. We studied the 13 most relevant polymorphisms, selected based on their functionality and allele frequency, in 6 genes essential for paclitaxel hepatic metabolism (*CYP2C8*, *CYP3A4*, *CYP3A5*) (Rahman, et al. 1994; Vaclavikova et al. 2004) and transport (*SLCO1B1, SLCO1B3* and *ABCB1*) (Gui, et al. 2008; Smith, et al. 2005; Sparreboom, et al. 1997; Walle and Walle 1998) (Figure 4) and studied their association with the paclitaxel accumulated dose causing grade 2 neurotoxicity in 118 patients treated with this drug. We found a statistically significant association between three cytochrome P450 functional genetic variants and paclitaxel neurotoxicity. This data reveals a possible genetic predisposition to undergo paclitaxel-induced neuropathy.

3.1. Treatment schedule and age modify paclitaxel neurotoxicity

Because paclitaxel neurotoxicity is dose-dependent, (Mielke, et al. 2003) we calculated the accumulated paclitaxel dose that produced clinically relevant neurotoxicity, rather than just evaluating the presence or lack of neurotoxicity at the end of the treatment. We confirmed that the weekly 80-90 mg/m^2 paclitaxel schedule, was a risk factor for the neurotoxicity (Argyriou, et al. 2008; Seidman, et al. 2008) and, although previous results concerning age are inconclusive (Akerley, et al. 2003; Argyriou, et al. 2006; Mielke et al. 2003), we found that patients below 50 years developed greater neuropathy (Figure 17).

3.2. CYP2C8 and CYP3A5 SNPs are associated with neurotoxicity

We found a statistically significant association for three CYP polymorphisms, *CYP2C8*3, CYP2C8* Haplotype C and *CYP3A5*3* (Table 12) with paclitaxel neurotoxicity. Initial *in vitro* studies for *CYP2C8*3* suggested a lower activity for this allele (Dai, et al. 2001; Soyama, et al. 2001), but more recent studies in healthy volunteers have demonstrated an increased metabolizing capacity (Aquilante, et al. 2008; Kirchheiner, et al. 2008; Niemi, et al. 2005; Niemi, et al. 2003). In contrast, *CYP2C8* haplotype C (Kirchheiner et al. 2008; Rodriguez-Antona, et al. 2008; Smith, et al. 2008) and *CYP3A5*3* (Kuehl et al. 2001) confer a reduced activity. Given that the polymorphisms conferring low

metabolizing capacity were associated with protection, while the allele with increased activity showed a higher neurotoxicity (Figure 18), and the effects of these polymorphisms were independent, we incorporated them in a single prediction model (risk alleles: rs11572080 A, rs1113129 G and rs776746 G; Table 12). As result of combining the three independent alleles, individuals can be classified in 7 groups depending on the number of risk alleles. While inheriting 5 and 6 risk alleles is infrequent in Caucasians, carrying 0 to 4 is relatively common (frequency > 3%), and consequently, variation at these three loci could explain a substantial proportion of paclitaxel-related neurotoxicity (Figure 19). In addition to the discussed polymorphisms, *CYP3A4*1B* showed a tendency towards increased neurotoxicity (P=0.057). However, *CYP3A4*1B* is in strong linkage disequilibrium with the functional *CYP3A5*1* allele, suggesting that this latter is the causal polymorphism (Kuehl et al. 2001). A recent study by Gandara *et al.* (Gandara, et al. 2009) on paclitaxel/carboplatin treated patients suggests that population-related pharmacogenetic factors could account for ethnic differences in patient outcomes. However, no differences on paclitaxel neurotoxicity could be observed between Asian and USA patients (Gandara et al. 2009). This could be in agreement with our results since the protective *CYP3A5*3* allele is more frequent in Caucasians than in Asians (Gandara et al. 2009; Kuehl et al. 2001), but *CYP2C8*3* risk allele is mainly Caucasian and could thus counteract the protective effect (Gandara et al. 2009; Rodriguez-Antona et al. 2008). The frequency of *CYP2C8* haplotype C in different ethnic groups is unclear (Rodriguez-Antona et al. 2008) but, at least in Caucasians, it seems to be associated with a lower paclitaxel-related neurotoxicity risk.

Contradictory results have been obtained regarding the impact of genetic variation on paclitaxel neurotoxicity (Green, et al. 2009; Marsh, et al. 2007; Sissung, et al. 2006). A large study by Marsh *et al.* (Marsh et al. 2007) did not find evidence of associations with neurotoxicity for important polymorphisms in paclitaxel transporters and relevant CYPs. On the contrary, a more recent study by Green *et al.* found a statistically significant association for *CYP2C8*3* allele with increased neurotoxicity risk (Green et al. 2009), which is coincident with our findings. The differences among these studies could be caused by the different experimental designs: Green *et al.* included a reduced number of patients with neurotoxicity as the primary endpoint of the study (Green et al. 2009; Sissung et al. 2006), whereas Marsh *et al.* (Marsh et al. 2007) included a large number of patients, however, the cumulative effect of paclitaxel neurotoxicity was not taken into account. Additionally, in the study focused on *ABCB1* polymorphisms by Sissung *et al.* (Sissung et al. 2006), no significant differences were found in 22 patients evaluated for

neurotoxicity. Some of the genetic markers analyzed in our study [e.g. *CYP2C8* Haplotype C with relevant functionality (Rodriguez-Antona et al. 2008)] were not included in the previous analyses. Additionally, in the present study, the neurotoxicity was analyzed taking into account the dose dependency with paclitaxel (i.e. the dose accumulated causing neurotoxicity grade 2), rather than just evaluating the presence or lack of neurotoxicity at the end of the treatment. Limitations of our study include the heterogeneity of the patients in form of tumor types and treatment regimens, and the inclusion of part of the patients in a retrospective basis. Nevertheless, these factors were considered in the statistical analysis without substantial changes of the results.

3.3. An alteration in paclitaxel metabolism could modify the neurotoxicity risk

It is important to note that in our study polymorphisms affecting paclitaxel metabolism were associated with an altered risk of paclitaxel neurotoxicity. This data suggests that changes in paclitaxel metabolism could modify the neurotoxicity risk. If these findings are confirmed, external factors modifying paclitaxel metabolism (e.g. enzyme inhibitors) could be used to reduce the patients' risk of toxicity. Cell death assays have shown that paclitaxel hydroxy-metabolites are less active than paclitaxel (Kumar, et al. 1995; Sparreboom, et al. 1995), but paclitaxel cytotoxicity increases in the presence of non-cytotoxic concentrations of 6α-hydroxypaclitaxel (Kang, et al. 2001). However, the effect of these molecules in neurons, which might be damaged through an alteration of neurotransmitter trafficking rather than mitosis blockage (Nakata and Yorifuji 1999; Theiss and Meller 2000; Yang, et al. 2009), has not been described. The concentrations of the hydroxy-metabolites and the active paclitaxel form (unbound paclitaxel) in plasma have been shown to be in the same range (Monsarrat, et al. 1998; Walle, et al. 1995), but the concentrations in the peripheral nervous cells, where the neurotoxic damage occurs, have not been determined. Alternatively, the metabolites might act through indirect mechanisms interfering with paclitaxel effect. Further studies addressing these points such as pharmacokinetic analysis taking into account the identified polymorphisms are needed to improve our knowledge of the mechanisms underlying the neurotoxicity.

In conclusion our study, with paclitaxel neurotoxicity as primary endpoint, provides evidence that *CYP2C8* Haplotype C and *CYP3A5*3* are associated with lower risk of paclitaxel-related neurotoxicity and *CYP2C8*3* with increased risk. These results warrant

independent validation in a larger population before exploiting the clinical applications. If confirmed, these genetic variants could be used to inform treatment selection, providing the basis for an individualized paclitaxel pharmacotherapy.

DISCUSIÓN

1. Bloque I: *CYP3A5 se expresa en próstata a altos niveles, pero está ausente en el tejido tumoral prostático*

Se han publicado resultados contradictorios relativos a la expresión de los CYP3A en próstata (Di Paolo, et al. 2005; Finnstrom et al. 2001; Fujimura, et al. 2009; Koch, et al. 2002; Moilanen et al. 2007; Murray, et al. 1995a; Stamey et al. 2001; Zhang et al. 2006). En este trabajo demostramos que sólo el CYP3A5 se expresa en niveles relevantes en las células basolaterales de la próstata y que su expresión muestra una gran variación entre las muestras debido al polimorfismo *CYP3A5*3*, (Kuehl et al. 2001). Un resultado destacable fue la ausencia de expresión del CYP3A5 en el tejido tumoral prostático. Las diferencias con otros estudios podrían ser debidas a anticuerpos de baja especificidad (Moilanen et al. 2007) y el uso de PCR no cuantitativa. Ya que la CYP3A5 no se expresa en las células tumorales, no cabe esperar que *CYP3A5*3* influya de una forma directa en la inactivación del docetaxel. Por otra parte, dado que este enzima cataliza la hidroxilacion de androgenos a metabolitos menos activos, los polimorfismos que alteren la expresión prostática del CYP3A5 podrían influir en el riesgo a desarrollar cáncer de próstata (Parnes, et al. 2005; Zhenhua, et al. 2005). La mayoría de los estudios de asociación realizados se han centrado en el polimorfismo *CYP3A4*1B*, que no causa cambios relevantes en la expresión del CYP3A4 (Rodriguez-Antona et al. 2005) y que no se expresa en la próstata. Los resultados positivos obtenidos podrían explicarse por el alto grado de desequilibrio de ligamiento existente en el locus *CYP3A* (Kuehl et al. 2001; Lee et al. 2003), en concreto, el 80% de los Caucásicos tienen simultáneamente los polimorfismos *CYP3A4*1B* y *CYP3A5*1* (Wojnowski, et al. 2002).

2. Bloque II: *La familia miR-200 regula la expresión de la β-tubulina III y se asocia con la respuesta al tratamiento y supervivencia libre de recaida de los pacientes*

La mayoría de los carcinomas de ovario se detectan en estadios avanzados, siendo la falta de respuesta al tratamiento y la recaída los dos factores que reducen sustancialmente la supervivencia. De ahí la importancia de identificar nuevos biomarcadores de pronóstico y predictivos de respuesta al tratamiento. Mediante un análisis *in sílico* identificamos sitios de unión para los microRNAs de la familia miR-200 en el 3' UTR de los isotipos de β-tubulina I, II y III. Por otra parte, un análisis de IHC en 72 muestras de cáncer ovario reveló que el isotipo I de β-tubulina se expresaba a altos niveles en todas las muestras, mientras que las clases II y III mostraban una importante variación que era mutuamente excluyente. La sobreexpresión tumoral del isotipo III se ha asociado anteriormente con mal pronóstico y falta de respuesta en varios tipos de tumores, incluyendo ovario (Ferrandina et al. 2006; Umezu et al. 2008). En nuestro estudio no encontramos esta asociación, probablemente debido al reducido número de muestras con alta expresión del isotipo III. Al comparar los resultados de la IHC con los

niveles de expresión de los miR-200, descubrimos que la familia miR-200 regulaba la expresión del isotipo III, pero no la de los isotipos I y II, sugiriendo que mecanismos alternativos determinan la expresión final de estos isotipos. Por otra parte, los niveles de expresión del miR-200c se asociaron de forma estadísticamente significativa con la respuesta al tratamiento (P=0.0027 con t-test; HR=0.70, 95%CI=0.50-0.98, P=0.037 en análisis multivariante). Esto sugiere que una baja expresión del miR-200c podría conducir al sobre-expresión del isotipo III y a una mayor resistencia a paclitaxel. Esta hipótesis se corrobora con el estudio *in vitro* de Cochrane *et al* (Cochrane et al. 2009). Los miR-141, miR-200c y miR-429 mostraron una tendencia de asociación con la recaída de las pacientes. Por lo tanto, proponemos que la familia miR-200 podría ser un marcador tanto de pronóstico como de respuesta a tratamiento con taxanos en cáncer de ovario.

3. Bloque III: *Polimorfismos en los Citocromos P450 2C8 y 3A5 se asocian con la neurotoxidad del paclitaxel*

En este estudio de farmacogenética estudiamos 13 polimorfismos relevantes en seis genes implicados en la eliminación del paclitaxel en 118 pacientes tratados con este fármaco y en los que se había valorado la neurotoxicidad. Confirmamos que la administración semanal del paclitaxel era más neurotóxica que el régimen de 21 días (Argyriou et al. 2008; Seidman et al. 2008), y también encontramos una mayor neurotoxicidad en los pacientes menores de 50 años. Al analizar el efecto de los polimorfismos, descubrimos que el Haplotipo C del *CYP2C8* y el *CYP3A5*3* conferían protección frente el riesgo de sufrir neurotoxicidad por paclitaxel (P=0.014 y P=0.012, respectivamente) mientras que el *CYP2C8*3* aumentaba el riesgo (P=0.032). Estudios anteriores muestran resultados contradictorios para estos polimorfismos (Green et al. 2009; Marsh et al. 2007; Sissung et al. 2006), hecho que podría deberse en parte a un bajo número de muestras y a no tener en cuenta la dependencia de la neurotoxicidad con la dosis acumulada de paclitaxel. Ya que el *CYP2C8*3* posee una actividad incrementada (Aquilante et al. 2008; Kirchheiner et al. 2008; Niemi et al. 2005; Niemi et al. 2003), y el *CYP2C8* haplotipo C (Rodriguez-Antona et al. 2008) y el *CYP3A5*3* (Kuehl et al. 2001) confieren una actividad reducida, los tres polimorfismos fueron considerados conjuntamente en un modelo predictivo de neurotoxicidad (HR= 1.64 95%CI=1.26-2.14; P=0.0003). De acuerdo a este modelo los individuos se dividen en siete grupos, según el número de alelos de riesgo que portan, y se puede estimar su riesgo individual a desarrollar neurotoxicidad por paclitaxel.

CONCLUSIONS

1. Only *CYP3A5* out of the four *CYP3A* human genes is expressed at high levels in the non-tumoral prostate tissue, specifically in the basolateral cells and at variable levels due to the *CYP3A5*3* polymorphism.

2. CYP3A5 could play an important endogenous role in the prostate and *CYP3A* polymorphisms, such as *CYP3A5*3, CYP3A5*6* or *CYP3A5*7,* could alter the metabolism of intra-prostatic androgens and influence prostate cancer risk.

3. Tumoral prostate tissue lacks CYP3A5 expression. This suggests that polymorphisms altering the activity of this enzyme cannot directly affect the efficacy of docetaxel treatment in prostate cancer.

4. A decreased miR-200 family expression results in up-regulation of β-tubulin class III in ovarian tumours.

5. miR-200c could be used as a biomarker for treatment efficacy in advanced ovarian cancer since it is associated with the response to paclitaxel-carboplatin treatment.

6. miR-200 family could be a prognostic factor in advanced ovarian carcinoma since it is associated with progression-free survival.

7. *CYP2C8* Haplotype C and *CYP3A5*3* are associated with lower risk and *CYP2C8*3* is associated with an increased risk of paclitaxel neurotoxicity.

8. According to these results a genetic model including the three polymorphisms could be applied to predict the individual risk of the patients to develop paclitaxel neurotoxicity, providing the basis for an individualized paclitaxel pharmacotherapy.

CONCLUSIONES

1. De los cuatro genes *CYP3A* humanos sólo el *CYP3A5* se expresa a altos niveles en el tejido prostático no-tumoral, específicamente en las células basolaterales y en cantidades variables dependiendo del polimorfismo *CYP3A5*3.*

2. El CYP3A5 podría jugar en la próstata un importante papel endógeno y los polimorfismos del *CYP3A,* como el *CYP3A5*3, CYP3A5*6* y *CYP3A5*7,* podrían alterar el metabolismo de los andrógenos intra-prostáticos e influir en el riesgo de desarrollar cáncer de próstata.

3. El tejido tumoral prostático no expresa CYP3A5. Esto sugiere que polimorfismos que alteran la actividad de este enzima no pueden afectar de forma directa la eficacia del tratamiento del cáncer de próstata con docetaxel.

4. Una disminución en la expresión de la familia miR-200 resulta en un aumento de la expresión de la β-tubulina clase III en tumores de ovario.

5. El miR-200c podría utilizarse como un biomarcador de eficacia de tratamiento en cáncer de ovario avanzado, ya que está asociado a la respuesta al esquema paclitaxel-carboplatino.

6. La familia miR-200 podría ser un factor pronóstico para el carcinoma de ovario avanzado, ya que está asociado a la supervivencia libre de recaída.

7. El haplotipo C del *CYP2C8* y el *CYP3A5*3* están asociados a un menor riesgo y el *CYP2C8*3* está asociado a un mayor riesgo de desarrollar neurotoxicidad por paclitaxel.

8. De acuerdo a estos resultados estos tres polimorfismos se podrían incluir en un único modelo genético para predecir el riesgo de desarrollar neurotoxicidad por paclitaxel, proporcionando así la base para una farmacoterapia individualizada del paclitaxel.

REFERENCES

Adam L, Zhong M, Choi W, Qi W, Nicoloso M, Arora A, Calin G, Wang H, Siefker-Radtke A, McConkey D, Bar-Eli M & Dinney C 2009 miR-200 expression regulates epithelial-to-mesenchymal transition in bladder cancer cells and reverses resistance to epidermal growth factor receptor therapy. *Clin Cancer Res* **15** 5060-5072.

Akasaka K, Maesawa C, Shibazaki M, Maeda F, Takahashi K, Akasaka T & Masuda T 2009 Loss of class III beta-tubulin induced by histone deacetylation is associated with chemosensitivity to paclitaxel in malignant melanoma cells. *J Invest Dermatol* **129** 1516-1526.

Akerley W, Herndon JE, Egorin MJ, Lyss AP, Kindler HL, Savarese DM, Sherman CA, Rosen DM, Hollis D, Ratain MJ & Green MR 2003 Weekly, high-dose paclitaxel in advanced lung carcinoma: a phase II study with pharmacokinetics by the Cancer and Leukemia Group B. *Cancer* **97** 2480-2486.

Aoki D, Oda Y, Hattori S, Taguchi K, Ohishi Y, Basaki Y, Oie S, Suzuki N, Kono S, Tsuneyoshi M, Ono M, Yanagawa T & Kuwano M 2009 Overexpression of class III beta-tubulin predicts good response to taxane-based chemotherapy in ovarian clear cell adenocarcinoma. *Clin Cancer Res* **15** 1473-1480.

Aquilante CL, Bushman LR, Knutsen SD, Burt LE, Rome LC & Kosmiski LA 2008 Influence of SLCO1B1 and CYP2C8 gene polymorphisms on rosiglitazone pharmacokinetics in healthy volunteers. *Hum Genomics* **3** 7-16.

Argyriou AA, Koltzenburg M, Polychronopoulos P, Papapetropoulos S & Kalofonos HP 2008 Peripheral nerve damage associated with administration of taxanes in patients with cancer. *Crit Rev Oncol Hematol* **66** 218-228.

Argyriou AA, Polychronopoulos P, Koutras A, Iconomou G, Gourzis P, Assimakopoulos K, Kalofonos HP & Chroni E 2006 Is advanced age associated with increased incidence and severity of chemotherapy-induced peripheral neuropathy? *Support Care Cancer* **14** 223-229.

Baffa R, Fassan M, Volinia S, O'Hara B, Liu CG, Palazzo JP, Gardiman M, Rugge M, Gomella LG, Croce CM & Rosenberg A 2009 MicroRNA expression profiling of human metastatic cancers identifies cancer gene targets. *J Pathol* **219** 214-221.

Baker SD, Verweij J, Cusatis GA, van Schaik RH, Marsh S, Orwick SJ, Franke RM, Hu S, Schuetz EG, Lamba V, Messersmith WA, Wolff AC, Carducci MA & Sparreboom A 2009 Pharmacogenetic pathway analysis of docetaxel elimination. *Clin Pharmacol Ther* **85** 155-163.

Berrieman HK, Lind MJ & Cawkwell L 2004 Do beta-tubulin mutations have a role in resistance to chemotherapy? *Lancet Oncol* **5** 158-164.

Bosch TM, Huitema AD, Doodeman VD, Jansen R, Witteveen E, Smit WM, Jansen RL, van Herpen CM, Soesan M, Beijnen JH & Schellens JH 2006 Pharmacogenetic screening of CYP3A and ABCB1 in relation to population pharmacokinetics of docetaxel. *Clin Cancer Res* **12** 5786-5793.

Burchell B 2003 Genetic variation of human UDP-glucuronosyltransferase: implications in disease and drug glucuronidation. *Am J Pharmacogenomics* **3** 37-52.

Cavaletti G, Bogliun G, Marzorati L, Zincone A, Piatti M, Colombo N, Franchi D, La Presa MT, Lissoni A, Buda A, Fei F, Cundari S & Zanna C 2004 Early predictors of peripheral neurotoxicity in cisplatin and paclitaxel combination chemotherapy. *Ann Oncol* **15** 1439-1442.

Cochrane DR, Spoelstra NS, Howe EN, Nordeen SK & Richer JK 2009 MicroRNA-200c mitigates invasiveness and restores sensitivity to microtubule-targeting chemotherapeutic agents. *Mol Cancer Ther*.

Croce CM 2009 Causes and consequences of microRNA dysregulation in cancer. *Nat Rev Genet* **10** 704-714.

Chan LM, Lowes S & Hirst BH 2004 The ABCs of drug transport in intestine and liver: efflux proteins limiting drug absorption and bioavailability. *Eur J Pharm Sci* **21** 25-51.

Chaudhry V, Rowinsky EK, Sartorius SE, Donehower RC & Cornblath DR 1994 Peripheral neuropathy from taxol and cisplatin combination chemotherapy: clinical and electrophysiological studies. *Ann Neurol* **35** 304-311.

Chen H, Hao J, Wang L & Li Y 2009 Coexpression of invasive markers (uPA, CD44) and multiple drug-resistance proteins (MDR1, MRP2) is correlated with epithelial ovarian cancer progression. *Br J Cancer* **101** 432-440.

Chevillard S, Pouillart P, Beldjord C, Asselain B, Beuzeboc P, Magdelenat H & Vielh P 1996 Sequential assessment of multidrug resistance phenotype and measurement of S-phase fraction as predictive markers of breast cancer response to neoadjuvant chemotherapy. *Cancer* **77** 292-300.

Cho WC 2007 OncomiRs: the discovery and progress of microRNAs in cancers. *Mol Cancer* **6** 60.

Dai D, Zeldin DC, Blaisdell JA, Chanas B, Coulter SJ, Ghanayem BI & Goldstein JA 2001 Polymorphisms in human CYP2C8 decrease metabolism of the anticancer drug paclitaxel and arachidonic acid. *Pharmacogenetics* **11** 597-607.
Dawood S 2009 Pharmacology, pharmacogenetics, and pharmacoepidemiology: three ps of individualized therapy. *Cancer Invest* **27** 809-815.

Dawood S & Leyland-Jones B 2009 Pharmacology and pharmacogenetics of chemotherapeutic agents. *Cancer Invest* **27** 482-488.

De Ligio JT, Velkova A, Zorio DA & Monteiro AN 2009 Can the status of the breast and ovarian cancer susceptibility gene 1 product (BRCA1) predict response to taxane-based cancer therapy? *Anticancer Agents Med Chem* **9** 543-549.

DeLoia JA, Zamboni WC, Jones JM, Strychor S, Kelley JL & Gallion HH 2008 Expression and activity of taxane-metabolizing enzymes in ovarian tumors. *Gynecol Oncol* **108** 355-360.

Dhaini HR, Thomas DG, Giordano TJ, Johnson TD, Biermann JS, Leu K, Hollenberg PF & Baker LH 2003 Cytochrome P450 CYP3A4/5 expression as a biomarker of outcome in osteosarcoma. *J Clin Oncol* **21** 2481-2485.

Di Paolo OA, Teitel CH, Nowell S, Coles BF & Kadlubar FF 2005 Expression of cytochromes P450 and glutathione S-transferases in human prostate, and the potential for activation of heterocyclic amine carcinogens via acetyl-coA-, PAPS- and ATP-dependent pathways. *Int J Cancer* **117** 8-13.

Diaz JF & Andreu JM 1993 Assembly of purified GDP-tubulin into microtubules induced by taxol and taxotere: reversibility, ligand stoichiometry, and competition. *Biochemistry* **32** 2747-2755.

Downie D, McFadyen MC, Rooney PH, Cruickshank ME, Parkin DE, Miller ID, Telfer C, Melvin WT & Murray GI 2005 Profiling cytochrome P450 expression in ovarian cancer: identification of prognostic markers. *Clin Cancer Res* **11** 7369-7375.

Eichelbaum M, Ingelman-Sundberg M & Evans WE 2006 Pharmacogenomics and individualized drug therapy. *Annu Rev Med* **57** 119-137.

Eisenhauer EA, ten Bokkel Huinink WW, Swenerton KD, Gianni L, Myles J, van der Burg ME, Kerr I, Vermorken JB, Buser K, Colombo N & et al. 1994 European-Canadian randomized trial of paclitaxel in relapsed ovarian cancer: high-dose versus low-dose and long versus short infusion. *J Clin Oncol* **12** 2654-2666.

Evans WE & McLeod HL 2003 Pharmacogenomics--drug disposition, drug targets, and side effects. *N Engl J Med* **348** 538-549.

Evans WE & Relling MV 1999 Pharmacogenomics: translating functional genomics into rational therapeutics. *Science* **286** 487-491.

Evans WE & Relling MV 2004 Moving towards individualized medicine with pharmacogenomics. *Nature* **429** 464-468.

Ferrandina G, Zannoni GF, Martinelli E, Paglia A, Gallotta V, Mozzetti S, Scambia G & Ferlini C 2006 Class III beta-tubulin overexpression is a marker of poor clinical outcome in advanced ovarian cancer patients. *Clin Cancer Res* **12** 2774-2779.
Finnstrom N, Bjelfman C, Soderstrom TG, Smith G, Egevad L, Norlen BJ, Wolf CR & Rane A 2001 Detection of cytochrome P450 mRNA transcripts in prostate samples by RT-PCR. *Eur J Clin Invest* **31** 880-886.

Fojo T & Menefee M 2007 Mechanisms of multidrug resistance: the potential role of microtubule-stabilizing agents. *Ann Oncol* **18 Suppl 5** v3-8.

Fujimura T, Takahashi S, Urano T, Kumagai J, Murata T, Takayama K, Ogushi T, Horie-Inoue K, Ouchi Y, Kitamura T, Muramatsu M, Homma Y & Inoue S 2009 Expression of cytochrome P450 3A4 and its clinical significance in human prostate cancer. *Urology* **74** 391-397.

Gandara DR, Kawaguchi T, Crowley J, Moon J, Furuse K, Kawahara M, Teramukai S, Ohe Y, Kubota K, Williamson SK, Gautschi O, Lenz HJ, McLeod HL, Lara PN, Jr., Coltman CA, Jr., Fukuoka M, Saijo N, Fukushima M & Mack PC 2009 Japanese-US Common-Arm Analysis of Paclitaxel Plus Carboplatin in Advanced Non-Small-Cell Lung Cancer: A Model for Assessing Population-Related Pharmacogenomics. *J Clin Oncol*.

Giannakakou P, Sackett DL, Kang YK, Zhan Z, Buters JT, Fojo T & Poruchynsky MS 1997 Paclitaxel-resistant human ovarian cancer cells have mutant beta-tubulins that exhibit impaired paclitaxel-driven polymerization. *J Biol Chem* **272** 17118-17125.

Green H, Soderkvist P, Rosenberg P, Mirghani RA, Rymark P, Lundqvist EA & Peterson C 2009 Pharmacogenetic studies of Paclitaxel in the treatment of ovarian cancer. *Basic Clin Pharmacol Toxicol* **104** 130-137.

Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, Vadas MA, Khew-Goodall Y & Goodall GJ 2008 The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol* **10** 593-601.

Gui C, Miao Y, Thompson L, Wahlgren B, Mock M, Stieger B & Hagenbuch B 2008 Effect of pregnane X receptor ligands on transport mediated by human OATP1B1 and OATP1B3. *Eur J Pharmacol* **584** 57-65.

Gurwitz D & Weizman A 2004 Personalized psychiatry: a realistic goal. *Pharmacogenomics* **5** 213-217.

Hardisson M-B, Sánchez, Sarrió, Suárez, Calero, Palacios 2003 Tissue microarray immunohistochemical expression analysis of mismatch repair (hMLH1 and hMSH2 genes) in endometrial carcinoma and atypical endometrial hyperplasia: relationship with microsatellite instability. *Modern Pathology* **16** 11.

Hari M, Yang H, Zeng C, Canizales M & Cabral F 2003 Expression of class III beta-tubulin reduces microtubule assembly and confers resistance to paclitaxel. *Cell Motil Cytoskeleton* **56** 45-56.

Hasegawa S, Miyoshi Y, Egawa C, Ishitobi M, Taguchi T, Tamaki Y, Monden M & Noguchi S 2003 Prediction of response to docetaxel by quantitative analysis of class I and III beta-tubulin isotype mRNA expression in human breast cancers. *Clin Cancer Res* **9** 2992-2997.

Heintz AP, Odicino F, Maisonneuve P, Quinn MA, Benedet JL, Creasman WT, Ngan HY, Pecorelli S & Beller U 2006 Carcinoma of the ovary. FIGO 6th Annual

Report on the Results of Treatment in Gynecological Cancer. *Int J Gynaecol Obstet* **95 Suppl 1** S161-192.

Henningsson A, Marsh S, Loos WJ, Karlsson MO, Garsa A, Mross K, Mielke S, Vigano L, Locatelli A, Verweij J, Sparreboom A & McLeod HL 2005 Association of CYP2C8, CYP3A4, CYP3A5, and ABCB1 polymorphisms with the pharmacokinetics of paclitaxel. *Clin Cancer Res* **11** 8097-8104.

Hopper-Borge E, Chen ZS, Shchaveleva I, Belinsky MG & Kruh GD 2004 Analysis of the drug resistance profile of multidrug resistance protein 7 (ABCC10): resistance to docetaxel. *Cancer Res* **64** 4927-4930.

Horwitz SB, Cohen D, Rao S, Ringel I, Shen HJ & Yang CP 1993 Taxol: mechanisms of action and resistance. *J Natl Cancer Inst Monogr* 55-61.
Horwitz SB, Lothstein L, Manfredi JJ, Mellado W, Parness J, Roy SN, Schiff PB, Sorbara L & Zeheb R 1986 Taxol: mechanisms of action and resistance. *Ann N Y Acad Sci* **466** 733-744.

Hu X, Macdonald DM, Huettner PC, Feng Z, El Naqa IM, Schwarz JK, Mutch DG, Grigsby PW, Powell SN & Wang X 2009 A miR-200 microRNA cluster as prognostic marker in advanced ovarian cancer. *Gynecol Oncol* **114** 457-464.

Huang S 2006 Membrane transporters and channels in chemoresistance and -sensitivity of tumor cells *Cancer letters* **239** 15.

Huisman C, van Tellingen, Beijnen, Schinkel 2005 MRP2 (ABCC2) transports taxanes and confers paclitaxel resistance and both processes are stimulated by probenecid. *International Journal of Cancer.* **116** 6.

Huisman MT, Chhatta AA, van Tellingen O, Beijnen JH & Schinkel AH 2005 MRP2 (ABCC2) transports taxanes and confers paclitaxel resistance and both processes are stimulated by probenecid. *Int J Cancer* **116** 824-829.

Hustert E, Haberl M, Burk O, Wolbold R, He YQ, Klein K, Nuessler AC, Neuhaus P, Klattig J, Eiselt R, Koch I, Zibat A, Brockmoller J, Halpert JR, Zanger UM & Wojnowski L 2001 The genetic determinants of the CYP3A5 polymorphism. *Pharmacogenetics* **11** 773-779.

Ingelman-Sundberg M 2008 Pharmacogenomic biomarkers for prediction of severe adverse drug reactions. *N Engl J Med* **358** 637-639.

Izutsu N, Maesawa C, Shibazaki M, Oikawa H, Shoji T, Sugiyama T & Masuda T 2008 Epigenetic modification is involved in aberrant expression of class III beta-tubulin, TUBB3, in ovarian cancer cells. *Int J Oncol* **32** 1227-1235.

Jemal A, Siegel R, Ward E, Hao Y, Xu J & Thun MJ 2009 Cancer statistics, 2009. *CA Cancer J Clin* **59** 225-249.

Kalow T, Meyer 2001 In *Pharmacogenomics*, p 17: CRC Press

Kang MH, Figg WD, Ando Y, Blagosklonny MV, Liewehr D, Fojo T & Bates SE 2001 The P-glycoprotein antagonist PSC 833 increases the plasma concentrations of 6alpha-hydroxypaclitaxel, a major metabolite of paclitaxel. *Clin Cancer Res* **7** 1610-1617.

Kaplan EL & Meier P 1958 Nonparametric estimation from incomplete observations. *J Am Stat Assoc* **53** 457-481.

Kapucuoglu N, Coban T, Raunio H, Pelkonen O, Edwards RJ, Boobis AR & Iscan M 2003 Expression of CYP3A4 in human breast tumour and non-tumour tissues. *Cancer Lett* **202** 17-23.

Kavallaris M, Burkhart CA & Horwitz SB 1999 Antisense oligonucleotides to class III beta-tubulin sensitize drug-resistant cells to Taxol. *Br J Cancer* **80** 1020-1025.
Kavallaris M, Kuo DY, Burkhart CA, Regl DL, Norris MD, Haber M & Horwitz SB 1997 Taxol-resistant epithelial ovarian tumors are associated with altered expression of specific beta-tubulin isotypes. *J Clin Invest* **100** 1282-1293.

Kimchi-Sarfaty C, Marple AH, Shinar S, Kimchi AM, Scavo D, Roma MI, Kim IW, Jones A, Arora M, Gribar J, Gurwitz D & Gottesman MM 2007 Ethnicity-related polymorphisms and haplotypes in the human ABCB1 gene. *Pharmacogenomics* **8** 29-39.

Kirchheiner J, Meineke I, Fuhr U, Rodriguez-Antona C, Lebedeva E & Brockmoller J 2008 Impact of genetic polymorphisms in CYP2C8 and rosiglitazone intake on the urinary excretion of dihydroxyeicosatrienoic acids. *Pharmacogenomics* **9** 277-288.

Knupfer H, Schmidt R, Stanitz D, Brauckhoff M, Schonfelder M & Preiss R 2004 CYP2C and IL-6 expression in breast cancer. *Breast* **13** 28-34.

Koch I, Weil R, Wolbold R, Brockmoller J, Hustert E, Burk O, Nuessler A, Neuhaus P, Eichelbaum M, Zanger U & Wojnowski L 2002 Interindividual variability and tissue-specificity in the expression of cytochrome P450 3A mRNA. *Drug Metab Dispos* **30** 1108-1114.

Koh Y, Kim TM, Jeon YK, Kwon TK, Hah JH, Lee SH, Kim DW, Wu HG, Rhee CS, Sung MW, Kim CW, Kim KH & Heo DS 2009 Class III beta-tubulin, but not ERCC1, is a strong predictive and prognostic marker in locally advanced head and neck squamous cell carcinoma. *Ann Oncol* **20** 1414-1419.

Korpal M, Lee ES, Hu G & Kang Y 2008 The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. *J Biol Chem* **283** 14910-14914.

Kuehl P, Zhang J, Lin Y, Lamba J, Assem M, Schuetz J, Watkins PB, Daly A, Wrighton SA, Hall SD, Maurel P, Relling M, Brimer C, Yasuda K, Venkataramanan R, Strom S, Thummel K, Boguski MS & Schuetz E 2001 Sequence diversity in CYP3A promoters and characterization of the genetic basis of polymorphic CYP3A5 expression. *Nat Genet* **27** 383-391.

Kumar G, Ray S, Walle T, Huang Y, Willingham M, Self S & Bhalla K 1995 Comparative in vitro cytotoxic effects of taxol and its major human metabolite 6 alpha-hydroxytaxol. *Cancer Chemother Pharmacol* **36** 129-135.

Leandro-Garcia LJ LS, Landa I, Montero-Conde C, Lopez-Jimenez E, Leton R, Cascon A, Robledo M, Rodriguez-Antona C Tumoral and tissue-specific expression of the major human β-tubulin isotypes. *Submitted for publication*.

Lee SJ, Usmani KA, Chanas B, Ghanayem B, Xi T, Hodgson E, Mohrenweiser HW & Goldstein JA 2003 Genetic findings and functional studies of human CYP3A5 single nucleotide polymorphisms in different ethnic groups. *Pharmacogenetics* **13** 461-472.

Leschziner GD, Andrew T, Pirmohamed M & Johnson MR 2007 ABCB1 genotype and PGP expression, function and therapeutic drug response: a critical review and recommendations for future research. *Pharmacogenomics J* **7** 154-179.

Li Y, VandenBoom TG, 2nd, Kong D, Wang Z, Ali S, Philip PA & Sarkar FH 2009 Up-regulation of miR-200 and let-7 by natural agents leads to the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells. *Cancer Res* **69** 6704-6712.

Lin YS, Dowling AL, Quigley SD, Farin FM, Zhang J, Lamba J, Schuetz EG & Thummel KE 2002 Co-regulation of CYP3A4 and CYP3A5 and contribution to hepatic and intestinal midazolam metabolism. *Mol Pharmacol* **62** 162-172.
Lipton RB, Apfel SC, Dutcher JP, Rosenberg R, Kaplan J, Berger A, Einzig AI, Wiernik P & Schaumburg HH 1989 Taxol produces a predominantly sensory neuropathy. *Neurology* **39** 368-373.

Livak KJ & Schmittgen TD 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25** 402-408.

Marsh S, Paul J, King CR, Gifford G, McLeod HL & Brown R 2007 Pharmacogenetic assessment of toxicity and outcome after platinum plus taxane chemotherapy in ovarian cancer: the Scottish Randomised Trial in Ovarian Cancer. *J Clin Oncol* **25** 4528-4535.

McLeod HL & Evans WE 2001 Pharmacogenomics: unlocking the human genome for better drug therapy. *Annu Rev Pharmacol Toxicol* **41** 101-121.

Mielke S, Mross K, Gerds TA, Schmidt A, Wasch R, Berger DP, Lange W & Behringer D 2003 Comparative neurotoxicity of weekly non-break paclitaxel infusions over 1 versus 3 h. *Anticancer Drugs* **14** 785-792.

Mielke S, Sparreboom A, Steinberg SM, Gelderblom H, Unger C, Behringer D & Mross K 2005 Association of Paclitaxel pharmacokinetics with the development of peripheral neuropathy in patients with advanced cancer. *Clin Cancer Res* **11** 4843-4850.

Miyoshi Y, Ando A, Takamura Y, Taguchi T, Tamaki Y & Noguchi S 2002 Prediction of response to docetaxel by CYP3A4 mRNA expression in breast cancer tissues. *Int J Cancer* **97** 129-132.

Modugno F, Knoll C, Kanbour-Shakir A & Romkes M 2003 A potential role for the estrogen-metabolizing cytochrome P450 enzymes in human breast carcinogenesis. *Breast Cancer Res Treat* **82** 191-197.

Moilanen AM, Hakkola J, Vaarala MH, Kauppila S, Hirvikoski P, Vuoristo JT, Edwards RJ & Paavonen TK 2007 Characterization of androgen-regulated expression of CYP3A5 in human prostate. *Carcinogenesis* **28** 916-921.

Monsarrat B, Chatelut E, Royer I, Alvinerie P, Dubois J, Dezeuse A, Roche H, Cros S, Wright M & Canal P 1998 Modification of paclitaxel metabolism in a cancer patient by induction of cytochrome P450 3A4. *Drug Metab Dispos* **26** 229-233.

Mozzetti S, Ferlini C, Concolino P, Filippetti F, Raspaglio G, Prislei S, Gallo D, Martinelli E, Ranelletti FO, Ferrandina G & Scambia G 2005 Class III beta-tubulin overexpression is a prominent mechanism of paclitaxel resistance in ovarian cancer patients. *Clin Cancer Res* **11** 298-305.

Murray GI, Pritchard S, Melvin WT & Burke MD 1995a Cytochrome P450 CYP3A5 in the human anterior pituitary gland. *FEBS Lett* **364** 79-82.

Murray GI, Taylor VE, McKay JA, Weaver RJ, Ewen SW, Melvin WT & Burke MD 1995b The immunohistochemical localization of drug-metabolizing enzymes in prostate cancer. *J Pathol* **177** 147-152.

Nakata T & Yorifuji H 1999 Morphological evidence of the inhibitory effect of taxol on the fast axonal transport. *Neurosci Res* **35** 113-122.

Nam EJ, Yoon H, Kim SW, Kim H, Kim YT, Kim JH, Kim JW & Kim S 2008 MicroRNA expression profiles in serous ovarian carcinoma. *Clin Cancer Res* **14** 2690-2695.

Nelson MR, Bacanu SA, Mosteller M, Li L, Bowman CE, Roses AD, Lai EH & Ehm MG 2009 Genome-wide approaches to identify pharmacogenetic contributions to adverse drug reactions. *Pharmacogenomics J* **9** 23-33.

Niemi M, Backman JT, Kajosaari LI, Leathart JB, Neuvonen M, Daly AK, Eichelbaum M, Kivisto KT & Neuvonen PJ 2005 Polymorphic organic anion transporting polypeptide 1B1 is a major determinant of repaglinide pharmacokinetics. *Clin Pharmacol Ther* **77** 468-478.

Niemi M, Leathart JB, Neuvonen M, Backman JT, Daly AK & Neuvonen PJ 2003 Polymorphism in CYP2C8 is associated with reduced plasma concentrations of repaglinide. *Clin Pharmacol Ther* **74** 380-387.

Ohishi Y, Oda Y, Basaki Y, Kobayashi H, Wake N, Kuwano M & Tsuneyoshi M 2007 Expression of beta-tubulin isotypes in human primary ovarian carcinoma. *Gynecol Oncol* **105** 586-592.

Omura GA 2008 Progress in gynecologic cancer research: the Gynecologic Oncology Group experience. *Semin Oncol* **35** 507-521.

Oyama T, Morita M, Isse T, Kagawa N, Nakata S, So T, Mizukami M, Ichiki Y, Ono K, Sugaya M, Uramoto H, Yoshimatsu T, Hanagiri T, Sugio K, Kawamoto T & Yasumoto K 2005 Immunohistochemical evaluation of cytochrome P450 (CYP) and p53 in breast cancer. *Front Biosci* **10** 1156-1161.

Ozols RF, Bundy BN, Greer BE, Fowler JM, Clarke-Pearson D, Burger RA, Mannel RS, DeGeest K, Hartenbach EM & Baergen R 2003 Phase III trial of carboplatin and paclitaxel compared with cisplatin and paclitaxel in patients with optimally resected stage III ovarian cancer: a Gynecologic Oncology Group study. *J Clin Oncol* **21** 3194-3200.

Paradiso A, Mangia A, Chiriatti A, Tommasi S, Zito A, Latorre A, Schittulli F & Lorusso V 2005 Biomarkers predictive for clinical efficacy of taxol-based chemotherapy in advanced breast cancer. *Ann Oncol* **16 Suppl 4** iv14-19.

Park SM, Gaur AB, Lengyel E & Peter ME 2008 The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. *Genes Dev* **22** 894-907.

Parnes HL, Thompson IM & Ford LG 2005 Prevention of hormone-related cancers: prostate cancer. *J Clin Oncol* **23** 368-377.

Petrylak DP, Tangen CM, Hussain MH, Lara PN, Jr., Jones JA, Taplin ME, Burch PA, Berry D, Moinpour C, Kohli M, Benson MC, Small EJ, Raghavan D & Crawford ED 2004 Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. *N Engl J Med* **351** 1513-1520.

Pirmohamed M, James S, Meakin S, Green C, Scott AK, Walley TJ, Farrar K, Park BK & Breckenridge AM 2004 Adverse drug reactions as cause of admission to hospital: prospective analysis of 18 820 patients. *BMJ* **329** 15-19.

Rahman A, Korzekwa KR, Grogan J, Gonzalez FJ & Harris JW 1994 Selective biotransformation of taxol to 6 alpha-hydroxytaxol by human cytochrome P450 2C8. *Cancer Res* **54** 5543-5546.

Raspaglio G, Filippetti F, Prislei S, Penci R, De Maria I, Cicchillitti L, Mozzetti S, Scambia G & Ferlini C 2008 Hypoxia induces class III beta-tubulin gene expression by HIF-1alpha binding to its 3' flanking region. *Gene* **409** 100-108.

Rodriguez-Antona C, Leskela S, Zajac M, Cuadros M, Alves J, Moneo MV, Martin C, Cigudosa JC, Carnero A, Robledo M, Benitez J & Martinez-Delgado B 2007 Expression of CYP3A4 as a predictor of response to chemotherapy in peripheral T-cell lymphomas. *Blood* **110** 3345-3351.

Rodriguez-Antona C, Niemi M, Backman JT, Kajosaari LI, Neuvonen PJ, Robledo M & Ingelman-Sundberg M 2008 Characterization of novel CYP2C8 haplotypes and their contribution to paclitaxel and repaglinide metabolism. *Pharmacogenomics J* **8** 268-277.

Rodriguez-Antona C, Sayi JG, Gustafsson LL, Bertilsson L & Ingelman-Sundberg M 2005 Phenotype-genotype variability in the human CYP3A locus as assessed by the probe drug quinine and analyses of variant CYP3A4 alleles. *Biochem Biophys Res Commun* **338** 299-305.

Rosell R, Scagliotti G, Danenberg KD, Lord RV, Bepler G, Novello S, Cooc J, Crino L, Sanchez JJ, Taron M, Boni C, De Marinis F, Tonato M, Marangolo M, Gozzelino F, Di Costanzo F, Rinaldi M, Salonga D & Stephens C 2003 Transcripts in pretreatment biopsies from a three-arm randomized trial in metastatic non-small-cell lung cancer. *Oncogene* **22** 3548-3553.

Rowinsky EK 1997 The development and clinical utility of the taxane class of antimicrotubule chemotherapy agents. *Annu Rev Med* **48** 353-374.

Rowinsky EK, Chaudhry V, Forastiere AA, Sartorius SE, Ettinger DS, Grochow LB, Lubejko BG, Cornblath DR & Donehower RC 1993a Phase I and pharmacologic study of paclitaxel and cisplatin with granulocyte colony-stimulating factor: neuromuscular toxicity is dose-limiting. *J Clin Oncol* **11** 2010-2020.

Rowinsky EK, Eisenhauer EA, Chaudhry V, Arbuck SG & Donehower RC 1993b Clinical toxicities encountered with paclitaxel (Taxol). *Semin Oncol* **20** 1-15.

Sale S, Sung R, Shen P, Yu K, Wang Y, Duran GE, Kim JH, Fojo T, Oefner PJ & Sikic BI 2002 Conservation of the class I beta-tubulin gene in human populations and lack of mutations in lung cancers and paclitaxel-resistant ovarian cancers. *Mol Cancer Ther* **1** 215-225.

Saloustros E, Mavroudis D & Georgoulias V 2008 Paclitaxel and docetaxel in the treatment of breast cancer. *Expert Opin Pharmacother* **9** 2603-2616.

Schiff PB, Fant J & Horwitz SB 1979 Promotion of microtubule assembly in vitro by taxol. *Nature* **277** 665-667.

Seidman AD, Berry D, Cirrincione C, Harris L, Muss H, Marcom PK, Gipson G, Burstein H, Lake D, Shapiro CL, Ungaro P, Norton L, Winer E & Hudis C 2008 Randomized phase III trial of weekly compared with every-3-weeks paclitaxel for metastatic breast cancer, with trastuzumab for all HER-2 overexpressors and random assignment to trastuzumab or not in HER-2 nonoverexpressors: final results of Cancer and Leukemia Group B protocol 9840. *J Clin Oncol* **26** 1642-1649.

Seve P & Dumontet C 2005 Chemoresistance in non-small cell lung cancer. *Curr Med Chem Anticancer Agents* **5** 73-88.

Seve P & Dumontet C 2008 Is class III beta-tubulin a predictive factor in patients receiving tubulin-binding agents? *Lancet Oncol* **9** 168-175.

Seve P, Isaac S, Tredan O, Souquet PJ, Pacheco Y, Perol M, Lafanechere L, Penet A, Peiller EL & Dumontet C 2005a Expression of class III {beta}-tubulin is predictive of patient outcome in patients with non-small cell lung cancer receiving vinorelbine-based chemotherapy. *Clin Cancer Res* **11** 5481-5486.

Seve P, Mackey J, Isaac S, Tredan O, Souquet PJ, Perol M, Lai R, Voloch A & Dumontet C 2005b Class III beta-tubulin expression in tumor cells predicts response and outcome in patients with non-small cell lung cancer receiving paclitaxel. *Mol Cancer Ther* **4** 2001-2007.

Shen T, Kuang YH, Ashby CR, Lei Y, Chen A, Zhou Y, Chen X, Tiwari AK, Hopper-Borge E, Ouyang J & Chen ZS 2009 Imatinib and nilotinib reverse multidrug resistance in cancer cells by inhibiting the efflux activity of the MRP7 (ABCC10). *PLoS One* **4** e7520.

Shimada T, Yamazaki H, Mimura M, Inui Y & Guengerich FP 1994 Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *J Pharmacol Exp Ther* **270** 414-423.

Sissung TM, Mross K, Steinberg SM, Behringer D, Figg WD, Sparreboom A & Mielke S 2006 Association of ABCB1 genotypes with paclitaxel-mediated peripheral neuropathy and neutropenia. *Eur J Cancer* **42** 2893-2896.

Smith HE, Jones JP, 3rd, Kalhorn TF, Farin FM, Stapleton PL, Davis CL, Perkins JD, Blough DK, Hebert MF, Thummel KE & Totah RA 2008 Role of cytochrome P450 2C8 and 2J2 genotypes in calcineurin inhibitor-induced chronic kidney disease. *Pharmacogenet Genomics* **18** 943-953.

Smith NF, Acharya MR, Desai N, Figg WD & Sparreboom A 2005 Identification of OATP1B3 as a high-affinity hepatocellular transporter of paclitaxel. *Cancer Biol Ther* **4** 815-818.

Smith NF, Marsh S, Scott-Horton TJ, Hamada A, Mielke S, Mross K, Figg WD, Verweij J, McLeod HL & Sparreboom A 2007 Variants in the SLCO1B3 gene: interethnic distribution and association with paclitaxel pharmacokinetics. *Clin Pharmacol Ther* **81** 76-82.

Somlo G, Doroshow JH, Synold T, Longmate J, Reardon D, Chow W, Forman SJ, Leong LA, Margolin KA, Morgan RJ, Jr., Raschko JW, Shibata SI, Tetef ML, Yen Y, Kogut N, Schriber J & Alvarnas J 2001 High-dose paclitaxel in combination with doxorubicin, cyclophosphamide and peripheral blood progenitor cell rescue in patients with high-risk primary and responding metastatic breast carcinoma: toxicity profile, relationship to paclitaxel pharmacokinetics and short-term outcome. *Br J Cancer* **84** 1591-1598.

Soyama A, Saito Y, Hanioka N, Murayama N, Nakajima O, Katori N, Ishida S, Sai K, Ozawa S & Sawada JI 2001 Non-synonymous single nucleotide alterations found in the CYP2C8 gene result in reduced in vitro paclitaxel metabolism. *Biol Pharm Bull* **24** 1427-1430.

Sparreboom A, Huizing MT, Boesen JJ, Nooijen WJ, van Tellingen O & Beijnen JH 1995 Isolation, purification, and biological activity of mono- and dihydroxylated paclitaxel metabolites from human feces. *Cancer Chemother Pharmacol* **36** 299-304.

Sparreboom A, van Asperen J, Mayer U, Schinkel AH, Smit JW, Meijer DK, Borst P, Nooijen WJ, Beijnen JH & van Tellingen O 1997 Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine. *Proc Natl Acad Sci U S A* **94** 2031-2035.

Stamey TA, Warrington JA, Caldwell MC, Chen Z, Fan Z, Mahadevappa M, McNeal JE, Nolley R & Zhang Z 2001 Molecular genetic profiling of Gleason grade 4/5 prostate cancers compared to benign prostatic hyperplasia. *J Urol* **166** 2171-2177.

Sullivan KF & Cleveland DW 1986 Identification of conserved isotype-defining variable region sequences for four vertebrate beta tubulin polypeptide classes. *Proc Natl Acad Sci U S A* **83** 4327-4331.

Swanton C, Nicke B, Schuett M, Eklund AC, Ng C, Li Q, Hardcastle T, Lee A, Roy R, East P, Kschischo M, Endesfelder D, Wylie P, Kim SN, Chen JG, Howell M, Ried T, Habermann JK, Auer G, Brenton JD, Szallasi Z & Downward J 2009 Chromosomal instability determines taxane response. *Proc Natl Acad Sci U S A* **106** 8671-8676.

Takano M, Otani Y, Tanda M, Kawami M, Nagai J & Yumoto R 2009 Paclitaxel-resistance conferred by altered expression of efflux and influx transporters for paclitaxel in the human hepatoma cell line, HepG2. *Drug Metab Pharmacokinet* **24** 418-427.

Theiss C & Meller K 2000 Taxol impairs anterograde axonal transport of microinjected horseradish peroxidase in dorsal root ganglia neurons in vitro. *Cell Tissue Res* **299** 213-224.

Tran A, Jullien V, Alexandre J, Rey E, Rabillon F, Girre V, Dieras V, Pons G, Goldwasser F & Treluyer JM 2006 Pharmacokinetics and toxicity of docetaxel: role of CYP3A, MDR1, and GST polymorphisms. *Clin Pharmacol Ther* **79** 570-580.

Trotti A, Byhardt R, Stetz J, Gwede C, Corn B, Fu K, Gunderson L, McCormick B, Morrisintegral M, Rich T, Shipley W & Curran W 2000 Common toxicity criteria: version 2.0. an improved reference for grading the acute effects of cancer treatment: impact on radiotherapy. *Int J Radiat Oncol Biol Phys* **47** 13-47.

Umezu T, Shibata K, Kajiyama H, Terauchi M, Ino K, Nawa A & Kikkawa F 2008 Taxol resistance among the different histological subtypes of ovarian cancer may

be associated with the expression of class III beta-tubulin. *Int J Gynecol Pathol* **27** 207-212.

Urano N, Fujiwara Y, Doki Y, Kim SJ, Miyoshi Y, Noguchi S, Miyata H, Takiguchi S, Yasuda T, Yano M & Monden M 2006 Clinical significance of class III beta-tubulin expression and its predictive value for resistance to docetaxel-based chemotherapy in gastric cancer. *Int J Oncol* **28** 375-381.

Vaclavikova R, Soucek P, Svobodova L, Anzenbacher P, Simek P, Guengerich FP & Gut I 2004 Different in vitro metabolism of paclitaxel and docetaxel in humans, rats, pigs, and minipigs. *Drug Metab Dispos* **32** 666-674.

Walle T, Walle UK, Kumar GN & Bhalla KN 1995 Taxol metabolism and disposition in cancer patients. *Drug Metab Dispos* **23** 506-512.

Walle UK & Walle T 1998 Taxol transport by human intestinal epithelial Caco-2 cells. *Drug Metab Dispos* **26** 343-346.

Westlind A, Malmebo S, Johansson I, Otter C, Andersson TB, Ingelman-Sundberg M & Oscarson M 2001 Cloning and tissue distribution of a novel human cytochrome p450 of the CYP3A subfamily, CYP3A43. *Biochem Biophys Res Commun* **281** 1349-1355.

Wojnowski L, Hustert E, Klein K, Goldammer M, Haberl M, Kirchheiner J, Koch I, Klattig J, Zanger U & Brockmoller J 2002 Re: modification of clinical presentation of prostate tumors by a novel genetic variant in CYP3A4. *J Natl Cancer Inst* **94** 630-631; author reply 631-632.

Wrighton SA & Stevens JC 1992 The human hepatic cytochromes P450 involved in drug metabolism. *Crit Rev Toxicol* **22** 1-21.

Yang H, Kong W, He L, Zhao JJ, O'Donnell JD, Wang J, Wenham RM, Coppola D, Kruk PA, Nicosia SV & Cheng JQ 2008a MicroRNA expression profiling in human ovarian cancer: miR-214 induces cell survival and cisplatin resistance by targeting PTEN. *Cancer Res* **68** 425-433.

Yang IH, Siddique R, Hosmane S, Thakor N & Hoke A 2009 Compartmentalized microfluidic culture platform to study mechanism of paclitaxel-induced axonal degeneration. *Exp Neurol* **218** 124-128.
Yang Z, Wu D, Bui T & Ho RJ 2008b A novel human multidrug resistance gene MDR1 variant G571A (G191R) modulates cancer drug resistance and efflux transport. *J Pharmacol Exp Ther* **327** 474-481.

Yvon AM, Wadsworth P & Jordan MA 1999 Taxol suppresses dynamics of individual microtubules in living human tumor cells. *Mol Biol Cell* **10** 947-959.

Zhang W, Shannon WD, Duncan J, Scheffer GL, Scheper RJ & McLeod HL 2006 Expression of drug pathway proteins is independent of tumour type. *J Pathol* **209** 213-219.

Zhenhua L, Tsuchiya N, Narita S, Inoue T, Horikawa Y, Kakinuma H, Kato T, Ogawa O & Habuchi T 2005 CYP3A5 gene polymorphism and risk of prostate cancer in a Japanese population. *Cancer Lett* **225** 237-243.

APPENDIX I

Publications derived from the thesis