Whole-exome sequencing reveals defective *CYP3A4* variants predictive of paclitaxel dose-limiting neuropathy

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Translational Relevance

Paclitaxel is a cytotoxic agent widely used for the treatment of many cancers. Treatment with this drug frequently results in peripheral sensory neuropathy that can seriously impact patients' quality of life. We and others have shown that variant alleles moderately decreasing the expression of genes involved in paclitaxel metabolism (i.e. *CYP2C8*3* or *CYP3A4*22*) are associated with paclitaxel-induced neuropathy. The identification of predictive genetic markers for paclitaxel-induced dose-limiting neuropathy could lead to individualized risk assessment, facilitating treatment decision-making and therefore being of great clinical value. In this study, by whole exome sequencing of severe paclitaxel-induced peripheral neuropathy patients, we confirm the earlier described implication of CYP3A4 in paclitaxel-induced neuropathy and find an association of *CYP3A4* defective variants with paclitaxel treatment modifications. This study emphasizes the need to screen for rare genetic variants in selected cohorts of patients and may provide a basis for paclitaxel treatment individualization.

ABSTRACT

Purpose: Paclitaxel, a widely-used chemotherapeutic drug, can cause peripheral neuropathies leading to dose reductions and treatment suspensions and decreasing the quality of life of patients. It has been suggested that genetic variants altering paclitaxel pharmacokinetics increase neuropathy risk, but the major causes of inter-individual differences in susceptibility to paclitaxel toxicity remain unexplained. We carried out a whole-exome sequencing (WES) study to identify genetic susceptibility variants associated with paclitaxel neuropathy.

Experimental Design: Blood samples from eight patients with severe paclitaxel-induced peripheral neuropathy were selected for WES. An independent cohort of 228 cancer patients with complete paclitaxel neuropathy data was used for variant screening by DHPLC and association analysis. HEK293 cells were used for heterologous expression and characterization of two novel CYP3A4 enzymes.

Results: WES revealed two patients with rare *CYP3A4* variants, a premature stop codon (*CYP3A4*20* allele) and a novel missense variant (*CYP3A4*25*, p.P389S) causing reduced enzyme expression. Screening for *CYP3A4* variants in the independent cohort revealed three additional *CYP3A4*20* carriers, and two missense variants exhibiting diminished enzyme activity (*CYP3A4*8* and the novel *CYP3A4*27* allele, p.L475V). Relative to *CYP3A4* wild-type patients, those carrying *CYP3A4* variants had more severe neuropathy (2- and 1.3-fold higher risk of neuropathy for loss-of-function and missense variants, respectively, P=0.045) and higher probability of neuropathy-induced paclitaxel treatment modifications (7- and 3-fold higher risk for loss-of-function and missense variants, respectively, $P=5.9x10^{-5}$).

Conclusion: This is the first description of a genetic marker associated with paclitaxel treatment modifications caused by neuropathy. *CYP3A4* defective variants may provide a basis for paclitaxel treatment individualization.

INTRODUCTION

Paclitaxel is an antimicrotubular agent widely used for the treatment of many solid tumors. Peripheral neuropathy is the major toxicity limiting the clinical utility of this drug (1, 2). The degree of neuropathy is highly variable among patients, and while some remain asymptomatic throughout treatment, those with severe neuropathy can require paclitaxel dose reductions and treatment suspension, and therefore receive potentially sub-optimal treatment. The most severe cases sustain long-term damage to the peripheral nerves, substantially reducing their quality of life (3).

Paclitaxel-induced neuropathy is dose-dependent, and there are various clinical conditions that have been suggested as risk factors, such as diabetes mellitus, chronic liver disease, alcoholism and previous neuropathies (4). Genetic variation has also been suggested as a factor influencing neuropathy risk, based on both genome-wide association studies (5, 6) and candidate gene approaches focused on paclitaxel pharmacokinetics (7). A correlation between the severity of the neuropathy and paclitaxel levels in plasma has been described (8) (9). In fact, it has been shown that common polymorphisms in the two genes encoding paclitaxel metabolizing enzymes in the liver, *CYP2C8* (*CYP2C8*3*) and *CYP3A4* (*CYP3A4*22*), are associated with a moderately increased risk of developing neuropathy during paclitaxel treatment (8, 10, 11). The identification of predictive genetic markers for paclitaxel-induced dose-limiting neuropathy could lead to individualized risk assessment, facilitating treatment decision-making and therefore being of great clinical value.

The newly developed whole-exome sequencing (WES) technology facilitates the identification of mutations and rare variants in exons and exon/intron boundaries that may potentially be implicated in disease and in extreme phenotypes (12-16). Thus, WES could be applied to unveil novel high-impact alleles of importance for inter-individual variation in drug metabolism and adverse drug effects. In this study, we identified a loss-of-function allele and

a novel missense variant in the *CYP3A4* gene among eight patients with severe paclitaxel-induced neuropathy. Further screening for *CYP3A4* variants in an independent patient cohort revealed additional loss-of-function and missense allele carriers. Patients with *CYP3A4* variants had higher risk of neuropathy and a large increased risk of paclitaxel dose reductions or treatment suspensions. These results highlight the fact that genetic variants that are rare in the general population might be more prevalent in patient groups developing adverse drug reactions and indeed constitute pharmacogenomic biomarkers of value for individualized therapy.

PATIENTS AND METHODS

Materials used

Dibenzylfluorescein (DBF), fluorescein, paclitaxel, 3ρ-hydroxypaclitaxel (3OH-P), NADP+, glucose-6-phosphate and yeast glucose-6-phosphate dehydrogenase were purchased from Sigma-Aldrich (Sigma-Aldrich Sweden AB, Stockholm, Sweden). Lipofectamine LTX/PLUS and cell medium were purchased from Invitrogen (Life Technologies Europe BV, Stockholm, Sweden). All solvents for high-performance liquid chromatography (HPLC) assay were obtained from Merck KGaA (Darmstadt, Germany).

Patients

Blood samples from eight breast cancer patients with severe paclitaxel-induced neuropathy were collected at Spanish Hospitals specifically for this study. All eight patients had developed grade sensory 3 neuropathy (NCI-CTC v4 http://www.eortc.be/services/doc/ctc/) during weekly paclitaxel treatment at a cumulative dose $\leq$ 800 mg/m^2. Furthermore, the neuropathy resulted in treatment suspensions or paclitaxel dose reductions and continued with at least grade 2 intensity for more than 18 months after termination of paclitaxel treatment (Table 1). Other causes of neuropathy, such as diabetes mellitus, alcoholism, hepatic diseases, AIDS and previous neuropathies, were ruled out. DNA from an independent cohort of 228 breast and ovarian cancer patients treated with paclitaxel and recruited in different Spanish hospitals from Madrid, starting on January 2011, was available for genetic analysis. The primary objective of this series was to study paclitaxel-induced peripheral neuropathy, for this reason each patient had a complete neuropathy assessment. For each patient, the following information was available: demographics, tumor characteristics, maximum sensory neuropathy grade during paclitaxel treatment, neuropathy evolution once paclitaxel treatment was ceased, cumulative dose of paclitaxel, and paclitaxel dose reductions and suspensions and their causes (Suppl. Table S1).

All cancer patients were over 18 years of age, had documented histological cancer neoplasia, a life expectancy of ≥12 weeks and ECOG performance status ≤2, adequate bone marrow, renal and hepatic function and no previous history of neuropathy, and had taken some form of contraception.

To ensure homogeneity in neuropathy grading across the different collaborating centers, a qualified nurse (L.S.) trained by a neurologist (G.G.) interviewed by telephone all patients included in the study in a systematic manner to determine severity of symptoms and impairment in activities of daily living (e.g. extension and intensity of paresthesia, sensitivity, strength in hands and feet) and evaluated the neuropathy grade according to NCI-CTC v4. The recruitment of patients and collection of samples was approved by local internal ethical review committees and all patients gave written informed consent to participate in the study.

Whole-exome sequencing (WES)

WES of DNA samples from the eight patients with extreme sensory neuropathy (Table 1) was carried out at the National Centre for Genomic Analysis (CNAG). DNA was isolated from peripheral blood using the FlexiGene DNA Kit (Qiagen) and quality control was performed according to electrophoresis and spectrophotometric measurements. The Covaris S2 System (Covaris) was used for DNA fragmentation and exome capture was performed using the SureSelect XT HumanAllExon 50Mb kit (Agilent Technology). Library size and concentration was determined using Bioanalyzer 2100 (Agilent Technology). Exome sequencing at a mean coverage >50x was performed using 75-bp paired-end technology in a HiSeq2000 (Illumina). Real-time image analysis and base calling was performed using Illumina's Real Time Analysis software version 1.6 using standard parameters. The GEM (http://algorithms.cnag.cat/wiki/The_GEM_library) and BFAST (17) programs were used to align the reads against the whole human genome (hg19 assembly). To identify single

nucleotide variants (SNVs) and insertion-deletions (indels) the SAMtools program was used (http://samtools.sourceforge.net). Variants were filtered to rule out those in genome regions with low mappability, those with a strand bias p-value<0.001 in at least one sample and those with low depth read (<15x), the alternative allele present in <20% of reads, and/ or the alternative alleles present only in forward or reverse reads.

CYP3A4 variant detection

The full *CYP3A4* coding region was amplified in the prospective cohort by PCR using specific primers (Suppl. Table S2). Screening for *CYP3A4* variants was performed using denaturing high-performance liquid chromatography (DHPLC), in the DNA WAVE system 4500 HT (Transgenomic™, Crewe, UK), equipped with a DNASep column (Transgenomic™, Crewe, UK). Sequencing of PCR products was performed on an ABI PRISM 3700 DNA Analyzer capillary sequencer (Applied Biosystems).

Genotyping was performed on 15 ng of genomic DNA using the KASPar SNP Genotyping System (Kbiosciences). All assays included DNA samples with known genotypes and negative controls. The Sequence Detection System ABI PRISM® 7900HT (Applied Biosystems) was used to determine fluorescence and for allele assignment.

CYP3A4 expression vectors and heterologous expression

The coding region of CYP3A4.1 cDNA (NM_017460.5) was cloned into pCMV5 at the XbaI and KpnI restriction enzyme sites, to generate pCYP3A4.1 plasmid. To introduce c.1165C>T and c.1423C>G variants in pCYP3A4-wt, we used the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technology) following the manufacturer's instructions. The correct sequence of *CYP3A4* variant plasmids (pCYP3A4-P389S and pCYP3A4-L475V) was confirmed by sequencing.

HEK293 cells were cultured in DMEM with 10% fetal bovine serum and 100 U/ml penicillin-streptomycin. Cells were transfected with pCYP3A4.1, pCYP3A4-P389S, pCYP3A4-L475V or empty pCMV5, together with human cytochrome b5-a (pCL-cytb5) using Lipofectamine LTX/PLUS, following the manufacturer's guidelines.

Western blot analysis

Cells were solubilized in RIPA buffer containing Complete Protease Inhibitor Cocktail (Roche Diagnostics). Cell lysates or microsomes, prepared as previously described (18) and containing an equal amount of total protein, were separated using 15% SDS-polyacrylamide gel (18). Membranes were probed with primary antibodies against CYP3A4 (α-hCYP3A4, 1:1000 (19)), human cytochrome b5a (α-cytb5a, 1:1000; Santa Cruz Biotech) and, as loading control, the housekeeping escort chaperone ERp29 (α-ERp29, 1:1000 (20)). The amount of expressed CYP3A4 apoproteins was calculated by densitometric analysis (Image Gauge, v. 4.0; Fujifilm) of western blot bands using a standard calibration curve based on CYP3A4 supersomes (BD Bioscience). To determine protein stability, 48 hours post-transfection HEK293 cells were exposed to 50 µM cycloheximide (CXM) for 8 hours. The CYP3A4 degradation rate was estimated by immunoblotting and further densitometric analysis of protein bands.

Determination of CYP3A4 enzyme activity

All incubations were conducted as previously described (21, 22) with minor modifications. Briefly, microsomal fractions corresponding to 160 µg of protein were mixed with different concentrations of dibenzylfluorescein in 50 mM potassium phosphate buffer (pH 7.4) and the reaction was initiated by adding a pre-warmed NADPH-regenerating system. Fluorescein formation was proportional to incubation time and concentration of microsomes.

After 60 min, formation of metabolites was measured in a SPECTRAmax Gemini microplate spectrofluorometer (Molecular Devices) and results were analyzed using SoftMax Pro5 software.

Statistical analysis

Michaelis-Menten constants K_m, V_{max} and intrinsic clearance ($CL_{int}= V_{max}/K_m$) were calculated by nonlinear regression analysis using GraphPad and statistical significance was assessed using a paired *t*-test. Association between neuropathy grade (ranked 0 to 3) and specific genetic groups was assessed using the Goodman and Kruskal Gamma-test. Association between treatment modifications (binary variable) and specific genetic groups was assessed using Fisher's exact test. In these analyses potential confounders were accounted for by stratification using the Mantel-Haenszel test. Pearson's correlation test was used to compare all genetic groups simultaneously (the genetic variable ranked in three groups: wild type 0, missense variants 1, loss-of-function variants 2) with neuropathy grade and treatment modifications. The analysis SPSS software package v.19 was used for all statistical analyses. P-values less than 0.05 were considered statistically significant.

RESULTS

Detection of *CYP3A4*20* and *CYP3A4* c.1165C>T (p.P389S) variants by WES in two patients with extreme paclitaxel neuropathy

We first screened for loss-of-function and missense variants in critical genes involved in paclitaxel pharmacokinetics (*CYP3A4, CYP2C8, ABCB1* and *SLCO1B3*) in eight patients with extreme sensory neuropathy, and identified two high-impact variants in *CYP3A4* (Suppl. Table S3). One was the *CYP3A4*20* allele, a rare deleterious indel causing a frameshift and premature stop codon (c.1461_1462insA; p.P488Tfs*494). The other was a missense variant (c.1165C>T, p.P389S) located in the highly conserved CYP β-helix 4; it had not been reported previously, and was given the name *CYP3A4*25* by the CYP allele nomenclature committee (www.cypalleles.ki.se). The patients with these variants were women treated with adjuvant FEC+T for breast cancer that upon paclitaxel treatment developed grade 3 neuropathy with loss of sensitivity in hands and feet, dysesthesia and clumsiness, and walking problems. The *CYP3A4*20* carrier had 2 paclitaxel dose reductions due to the neuropathy and more than 40 months after paclitaxel treatment the symptoms persisted with improvement to grade 2. The *CYP3A4*25* carrier had suspension of paclitaxel treatment after cycle 6 due to the neuropathy and 19 months after paclitaxel treatment symptoms had decreased to grade 2 neuropathy; 25 months after paclitaxel treatment the patient did not report neuropathy symptoms. Sanger sequencing confirmed the presence of both variants in heterozygosity (Fig. 1).

In the *CYP2C8* gene, encoding the other CYP enzyme metabolizing paclitaxel, and in *SLCO1B3* and *ABCB1*, encoding the uptake and efflux paclitaxel transporters, respectively, we only detected previously described missense variants, none of which were predicted to affect protein function. In addition, with the exception of *CYP3A4*, the frequency of most variants in the patients with extreme neuropathy was similar to that reported in the general

population (Suppl. Table S3 shows coding polymorphisms and the regulatory intronic *CYP3A4*22*). Thus, we selected the *CYP3A4* gene for further study.

Screening for *CYP3A4* variants in a cohort of paclitaxel-treated patients

To determine whether additional *CYP3A4* coding variants are carried by patients with paclitaxel-induced neuropathy, we examined by DHPLC an independent cohort of 228 cancer patients treated with the drug (Suppl. Table S1). We detected three additional patients carrying the *CYP3A4*20* allele, another patient with *CYP3A4*8* allele (c.389G>A, p.R130Q) and another one with a novel *CYP3A4* missense variant (c.1423C>G, p.L475V, named *CYP3A4*27*). The characteristics of the patients carrying *CYP3A4* coding variants are shown in Table 2.

Stability and enzymatic activity of CYP3A4.25 and CYP3A4.27

The *CYP3A4*20* allele has been shown to encode a non-functional enzyme (23) and the *CYP3A4*8* allele was shown to cause a decreased CYP3A4 activity (24), but no functional data exist on the novel missense variants *CYP3A4*25* and *CYP3A4*27*. HEK293 cells transiently expressing CYP3A4-P389S or CYP3A4-L475V (CYP3A4.25 and CYP3A4.27, respectively) showed substantially lower amounts of protein compared to CYP3A4-wild type (CYP3A4.1) (Fig. 2). The level of the CYP3A4.27 protein in the expression system was estimated to be 10% of the corresponding expression of CYP3A4.1. The level of CYP3A4.25 was also relatively low, about 40% of the CYP3A4.1 levels and treatment of the cells with the protein synthesis inhibitor cycloheximide confirmed that the P389S substitution in CYP3A4.25 caused an increased rate of degradation (Suppl. Fig. S1). By analyzing the mRNA levels it was found that the levels were slightly lower (about 80-70% of the control) for the mutant variants as compared to CYP3A4.1 in the expression system (data not shown).

Analyses of catalytic activities of the variant enzymes using dibenzylfluorescein as a CYP3A4 substrate revealed a similar Km value for CYP3A4.27 (Km: 7.4 ± 1.8 µM) as compared to CYP3A4.1 whereas the Km value for CYP3A4.25 was somewhat higher (Km: 31.7 ± 2.8 µM). The true Vmax was difficult to determine because of low expression of the variant proteins (data not shown). We conclude that both CYP3A4.25 and CYP3A4.27 have decreased stability in the expression system used.

CYP3A4 variants are associated with an increased risk of neuropathy and paclitaxel treatment modifications

Thus, in a total of 236 patients, composed of eight WES-studied patients and 228 patients used for *CYP3A4* screening, four carried a loss-of-function variant (*CYP3A4*20*) and three carried rare missense variants giving rise to decreased enzymatic activity (*CYP3A4*25*, *CYP3A4*27* and *CYP3A4*8*) (Table 2).

The patients with *CYP3A4* loss-of-function variants and patients with missense variants showed a 2- and 1.3-fold increased risk of grade 3 neuropathy, respectively, when compared with wild-type *CYP3A4* patients (Fig. 3A). The difference in neuropathy grade was significantly different between patients with loss-of-function variants and wild-type homozygotes (P=0.042), and including missense variants in the analysis only minimally changed the P-value (P=0.045). Furthermore, 14% of patients with paclitaxel dose reductions or treatment suspensions due to neuropathy carried *CYP3A4* variants, and a 7- and 3-fold increased risk of treatment changes was observed in patients with loss-of-function and missense variants, respectively, when compared with wild-type *CYP3A4* patients (Fig. 3B). This increased risk of treatment modifications for patients with genetically decreased *CYP3A4* activity was statistically significant ($P=5.8x10^{-3}$ for loss-of-function variants, $P=5.9x10^{-5}$ when missense variants were included in the analysis). When all paclitaxel treatment modifications

(i.e. including those due to reasons other than neuropathy) were considered, *CYP3A4* variants were still associated with an increased risk of dose changes ($P=7.7x10^{-3}$).

Tumor type was not associated with the neuropathy, but conditions considered to be neuropathy risk factors (diabetes, high alcohol intake, restless-legs-syndrome) were significantly associated with neuropathy grade (Suppl. Fig. S2A). Cumulative paclitaxel dose was associated with treatment modifications, as expected, since these result in lower cumulative doses (Suppl. Fig. S2D). Concerning *CYP3A4*22* allele, we found a trend towards higher treatment modifications in carriers of this variant (P=0.066), however, no statistically significant differences were obtained for neuropathy grade and treatment modifications due to neuropathy (Suppl. Fig. S3). Accounting for neuropathy risk factors, cumulative paclitaxel dose or *CYP3A4*22* allele did not substantially change the association observed for *CYP3A4* variants.

DISCUSSION

Paclitaxel peripheral neuropathy affects a large number of patients and can lead to treatment modifications (25). Most patients recover from the neuropathy, but long-term nerve damage can also occur, compromising the quality of life of these patients. The extent of paclitaxel exposure is associated with the severity of the neuropathy (8, 9), and paclitaxel elimination is mediated by CYP2C8, CYP3A4, OATP1B3 and P-glycoprotein (7, 26, 27). Thus, alterations in the activity of these proteins could decrease drug elimination and consequently increase toxicity risk (e.g. *CYP2C8*3* (10, 11); *CYP3A4*22* (8, 28-30) or rs1045642 in *ABCB1* (31)).

The selection of phenotypic outliers has been shown to be an effective strategy to identify genetic variants associated with diseases and drug outcomes (13, 14, 23, 32), and when combined with massive parallel DNA sequencing (33) it is a powerful method to detect low-frequency susceptibility variants (16, 34, 35). Consequently, we designed a study to identify genetic variants in patients with severe paclitaxel-induced neuropathy. Chemotherapy-induced neurotoxicity studies are challenging due to subjectivity of the common toxicity scales and the difficult application of more accurate neuropathy scales across multiple centers (36). In this study neuropathy was assessed in a systematic manner in all collaborating centers, and for the identification of extreme-phenotype patients, not only the severity of symptoms during treatment, but also modifications of treatment regimen and long-lasting disabilities were taken into account.

WES revealed two rare high-impact variants in *CYP3A4* (*CYP3A4*20* and *CYP3A4*25*). *CYP3A4*20* is an indel leading to a premature stop codon previously described in one individual with impaired elimination of CYP3A4 substrates (23) and the novel CYP3A4.25 (P389S) protein had decreased stability and reduced amounts of apoprotein in the HEK293 expression system (Fig. 2A and Suppl. Fig. S1). In an independent cohort we found

three more carriers of *CYP3A4*20*, and two carriers of missense variants, CYP3A4.8, described to have diminished activity (24), and CYP3A4.27, here found to be less expressed in comparison to CYP3A4.1 (Fig. 2B). In total, 3% of the Spanish patients carried *CYP3A4* defective variants, suggesting that these could explain part of CYP3A4 variability. Although *CYP3A4*20* allele is present in Spain but has low frequency in most European, Asian and African populations (37), alternative *CYP3A4* defective variants might be relevant in other populations. In this respect, 3% of European Americans and 2% of African Americans seem to carry potentially defective *CYP3A4* allele variants (loss-of-function or missense variants likely damaging by Polyphen; see Exome Variant Server database).

Patients carrying loss-of-function *CYP3A4* variants had a significantly higher risk of neuropathy and paclitaxel treatment modifications, when compared with wild-type *CYP3A4* patients (P=0.042 and P=5.8x10^{-3}, respectively; Fig. 3), and carriers of missense variants showed an intermediate phenotype, concordant with a decreased but not abolished CYP3A4 activity. For *CYP3A4*22* allele, we only detected a trend towards increased treatment modifications, which might reflect a lower effect of this variant on paclitaxel metabolism and/ or a low statistical power due to the small number of *CYP3A4*22* carriers (Suppl. Fig. S3). Four out of the 29 patients with paclitaxel treatment modifications due to neuropathy carried *CYP3A4* defective variants (missense or loss-of-function), indicating that genetic testing of *CYP3A4* before treatment, would have a very high specificity (99%) but poor sensitivity (14%). If pathologic risk factors were also taken into consideration, the sensitivity would increase, and an estimated 27% of patients carrying *CYP3A4* defective variants or with preexisting conditions associated with neuropathy, would require treatment modification due to severe neuropathy upon paclitaxel chemotherapy. Paclitaxel-induced neuropathy is a multifactorial and polygenic trait, and additional genetic variants, some yet to be identified, will further improve the predictive power of genetic testing. In this regard, Supplementary

Table S4 shows genetic variants that have been described as moderate risk factors for paclitaxel-induced neuropathy (*CYP3A4*22*, *CYP2C8*3*, *EPHA5*-rs7349683 and *XKR4*-rs4737264, the latter two identified in a meta-analysis of genome-wide association studies (5, 6)). Thus, the identification of genetic variants and physiopathologic risk factors predictive of paclitaxel-induced neuropathy, may provide a basis on which to individualize this treatment. It is important to highlight that previous reports have only identified markers associated to neuropathy grade, but not with neuropathies resulting in treatment modifications. In fact, this is the first study describing a marker associated with paclitaxel dose-limiting neuropathy.

In summary, in this study we found an over-representation of defective *CYP3A4* variants in patients with paclitaxel treatment modifications and in those with high-grade paclitaxel-induced neuropathy. This supports and confirms the earlier described implication of CYP3A4 in paclitaxel-induced neuropathy. These results emphasize the need to screen for rare genetic variants in selected cohorts of patients.

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FIGURE LEGENDS

Figure 1. Coding variants identified by WES in *CYP3A4*. Sanger sequencing confirming the presence of **A**) *CYP3A4*20* (c.1461_1462insA) and **B)** *CYP3A4* c.1165 C>T variant alleles. The blue-highlighted regions indicate the position of the nucleotide change. **C)** Segment of CYP3A4 protein sequence alignment and predicted secondary structure. Proline 389, marked in red, is highly conserved across species. "*" indicates positions with a single, fully conserved residue, ":" indicates amino acids with very similar properties; "." indicates amino acids with mildly similar properties.

Figure 2. Expression of CYP3A4.25 and CYP3A4.27. Lysates from transfected HEK293 cells were immunoblotted with antibodies for CYP3A4 and ERp29, the latter as a loading control. A representative image is shown for **A)** CYP3A4.25 and **B)** CYP3A4.27, with samples loaded in triplicates (duplicates for mock transfected cells). Densitometric analysis estimated that CYP3A4 protein expression was 40% and 10% of the corresponding level of CYP3A4.1, for CYP3A4.25 and CYP3A4.27, respectively.

Figure 3. *CYP3A4* defective variants confer an increased risk of paclitaxel-induced neuropathy and treatment modifications. A) Neuropathy grade was compared among patients with different *CYP3A4* activity. All four patients with loss-of-function variants (100%), two out of three patients (67%) with missense variants, and 116 out of 229 patients (51%) with wild-type *CYP3A4* had grade 3 sensory neuropathy. **B)** Treatment modifications due to neuropathy were compared among patients with different *CYP3A4* activity. Three out of 4 patients (75%) with loss-of-function variants, 1 out of 3 patients (33%) with missense variants, and 25 out of 229 patients (11%) with wild-type *CYP3A4* had treatment modifications. As described in Materials and Methods, the Goodman and Kruskal's gamma

test and Fisher exact test were used to assess association with neuropathy grade (γ=1) and treatment modifications. To perform an analysis including simultaneously all variants categorized according to CYP3A4 activity (loss-of-function, missense and wild type) Pearson correlation test was used. Treatment modif PN, treatment modifications due to peripheral neuropathy.

TABLES

Table 1. Characteristics of the eight patients included in WES.

Characteristic	N*
Age (y)	
Median (min-max)	57 (39-79)
Tumor stage	
I	3
II	3
III	1
IV	1
First line chemotherapy treatment	
FEC+T[a]	5
FEC+T+H[b]	1
FEC75+T80[c]	1
T+H[d]	1
Nr. chemotherapy cycles	
(min-max)	(5-8)
Paclitaxel dose at sensory grade 3 neuropathy (mg/m^2)	
Median (min-max)	750 (400-800)
Duration of sensory neuropathy grade 2-3[e] (months)	
Median (min-max)	38 (20-66)
Treatment modifications due to neuropathy [f]	
Treatment suspension	4
Dose reduction	4

* Unless otherwise indicated

[a] FEC+T: 5-fluorouracil 600 mg/m^2, epirubicin 90 mg/m^2 and cyclophosphamide 600 mg/m^2, every 21 days, followed by paclitaxel 100 mg/m^2, every 7 days.

[b] FEC+T+H: 5-fluorouracil 600 mg/m^2, epirubicin 90 mg/m^2 and cyclophosphamide 600 mg/m^2, every 21 days, followed by paclitaxel 100 mg/m^2 plus herceptin (6 mg/kg loading dose; 2 mg/kg subsequent doses), every 7 days.

[c] FEC75+T80: 5-fluorouracil 600 mg/m^2, epirubicin 75 mg/m^2 and cyclophosphamide 600 mg/m^2, every 21 days, followed by paclitaxel 80 mg/m^2, every 7 days.

[d] T+H: paclitaxel 80 mg/m^2 plus herceptin (4 mg/kg loading dose; 2 mg/kg subsequent doses), every 7 days.

[e] Duration of grade 2 or 3 sensorial neuropathy after finishing paclitaxel treatment.

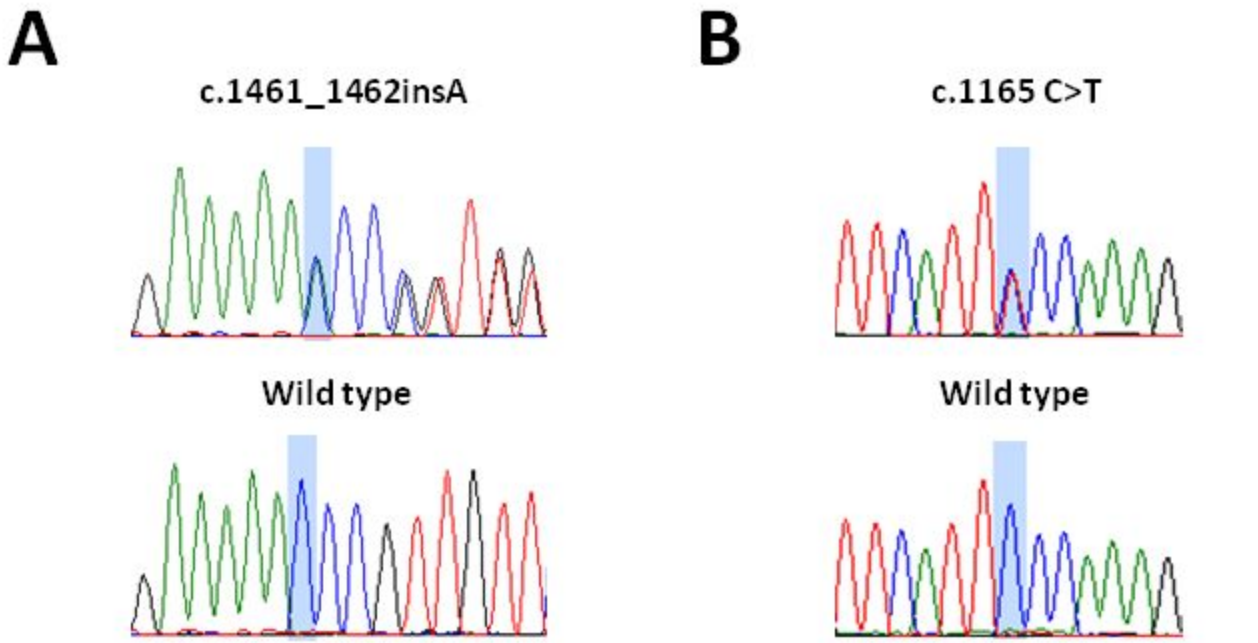
[f] When in the same patient paclitaxel dose was reduced and later on paclitaxel treatment was suspended, the patient is included in the table as "treatment suspension".

Table 2. Characteristics of patients with *CYP3A4* defective variants.

Patient	*CYP3A4* variant allele effect	*CYP3A4* genotype	Detection technique[a]	Neuropathy grade[b]	Total nr. cycles	Treatment modifications[c]	Time with neuropathy (months)[d]
11S1213	Loss-of-function	*1/ *20	WES	3	8	Red (at cycles 4 & 8)	(40)
11S919		*1/ *20	DHPLC	3	8	Red (at cycle 4)	(9)
13S812		*1/ *20	DHPLC	3	12	Red (at cycle 9)	(10)
13S120		*1/ *20	DHPLC	3	11	No	16
11S872	Decr. activity	*1/ *25	WES	3	6	Susp (after cycle 6)	19
12S513		*1/ *27	DHPLC	3	8	No	(19)
11S918		*1/ *8	DHPLC	1	12	No	6

[a] Confirmation of variants detected by DHPLC was performed by Sanger sequencing.
[b] Maximum sensory neuropathy grade during paclitaxel treatment (NCI-CTC v4).
[c] Modifications due to neuropathy. Red, reduction of the paclitaxel dose; Susp, suspension of paclitaxel treatment.
[d] Duration of neuropathy after finishing paclitaxel treatment. For cases for which the neuropathy was ongoing or further evaluation could not be performed because the patient died, the duration is written in parenthesis.

Figure 1

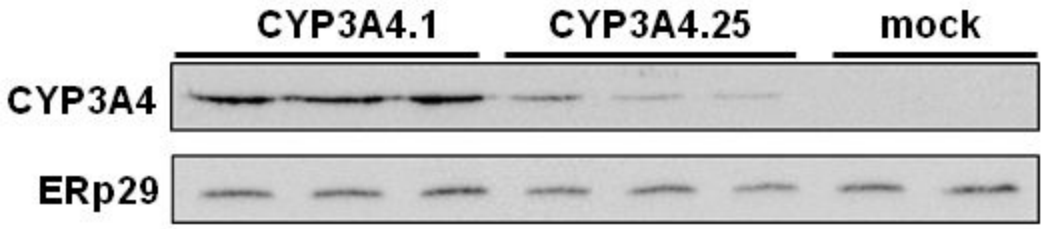


C


```
CYP3A4 Homo sapiens          LRLFPIAMRLERVCKKDVEINGMFIPKGVVVMIPSYALHRDPKY
Cyp3a4 Pan troglodytes       LRLFPVAMRLERVCKKDVEINGMFIPKGVVVMIPSYALHRDPKY
CYP3A4 Xenopus tropicalis    LRLYPTAIRLERVSKKDVEINGVFIPKGTVVMIPIYPLHRNPEY
Cyp3a59 Mus musculus         LRLFPPAGRLERVSKQNVEINGVSIPKGIVTLIPAYVLQRDPEY
CYP3C4 Danio rerio           MRLLPTAPRLERSAKKTVVINGLTIPEGTLVGIPTYVLSHDPDI
                             :** * * **** .*: * ***: **:* :. ** * * ::*.
```

Figure 2

A



B

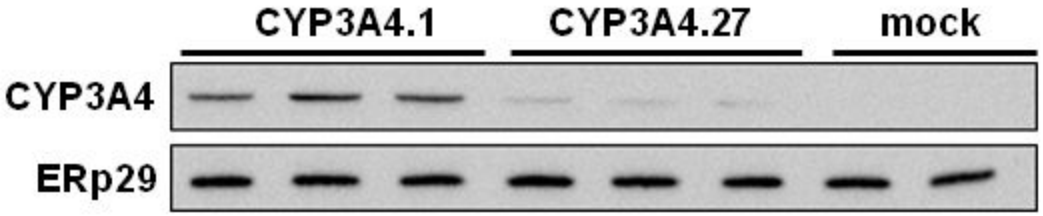
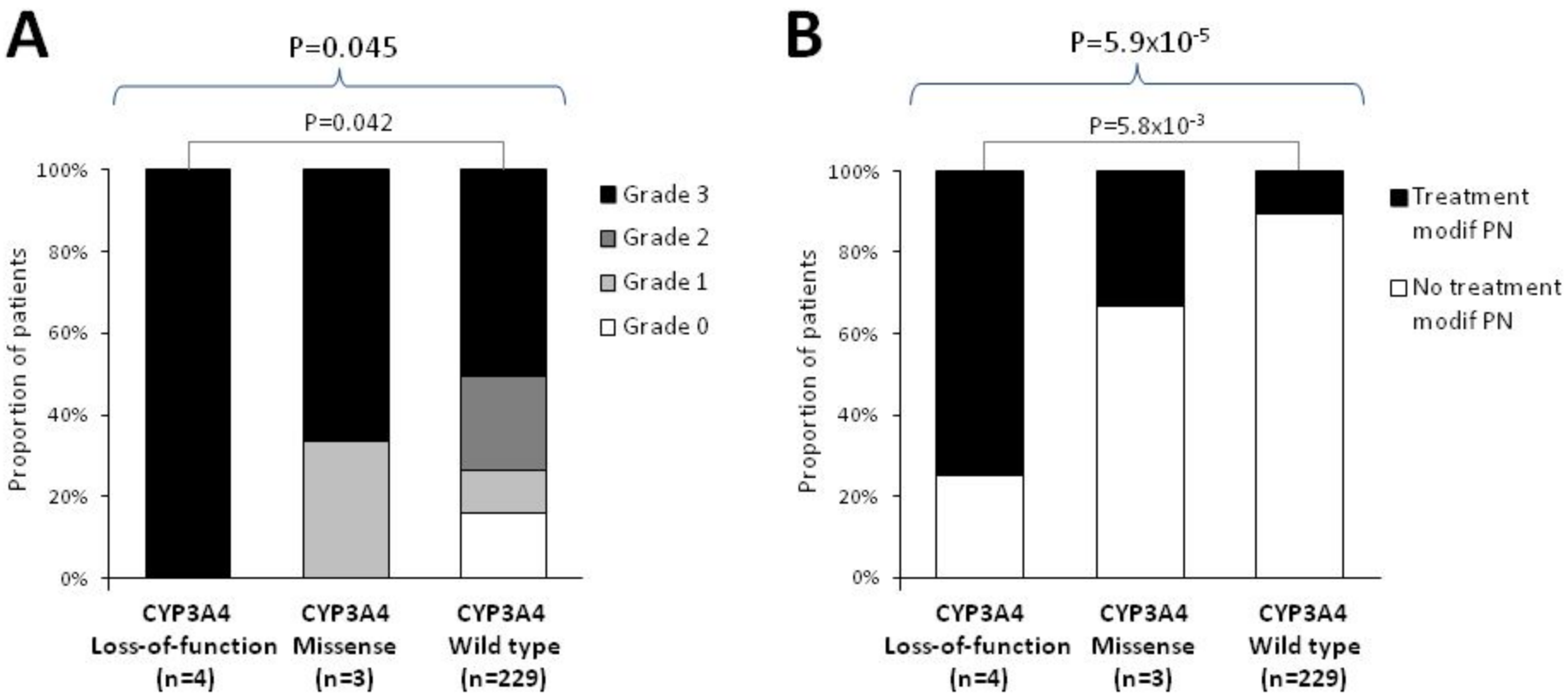


Figure 3



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Whole-exome sequencing reveals defective CYP3A4 variants predictive of paclitaxel dose-limiting neuropathy

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