Targeted sequencing reveals low-frequency variants in *EPHA* genes as markers of paclitaxel-induced peripheral neuropathy

Authors: María Apellániz-Ruiz[1], Héctor Tejero[2], Lucía Inglada-Pérez[1,3], Lara Sánchez-Barroso[1], Gerardo Gutiérrez-Gutiérrez[4], Isabel Calvo[5,6], Beatriz Castelo[7], Andrés Redondo[7], Jesus García-Donás[8], Nuria Romero-Laorden[8], María Sereno[9], María Merino[9], María Currás-Freixes[1], Cristina Montero-Conde[1], Veronika Mancikova[1], Elisabeth Åvall-Lundqvist[10], Henrik Green[11,12], Fátima Al-Shahrour[2], Alberto Cascón[1,3], Mercedes Robledo[1,3], Cristina Rodríguez-Antona[1,3]

Affiliations:

[1] Hereditary Endocrine Cancer Group, Spanish National Cancer Research Centre (CNIO), Madrid, Spain

[2] Translational Bioinformatics Unit, Spanish National Cancer Research Centre, Madrid, Spain

[3] ISCIII Center for Biomedical Research on Rare Diseases (CIBERER), Madrid, Spain

[4] Neurology Section, Hospital Universitario Infanta Sofía, Madrid, Spain

[5] Medical Oncology Department, Hospital Montepríncipe, Madrid, Spain

[6] Medical Oncology Department, Centro Integral Oncológico Clara Campal, Madrid, Spain

[7] Medical Oncology Department, Hospital Universitario La Paz, Madrid, Spain

[8] Gynecological and Genitourinary Tumors Programme, Centro Integral Oncológico Clara Campal, Madrid, Spain

[9] Medical Oncology Department, Hospital Universitario Infanta Sofía, Madrid, Spain

[10] Department of Oncology and Department of Clinical and Experimental Medicine, Linköpings Universitet, Linköping, Sweden

[11] Clinical Pharmacology, Division of Drug Research, Department of Medical and Health Sciences, Faculty of Health Sciences, Linköpings Universitet, Linköping, Sweden

[12] Department of Forensic Genetics and Forensic Toxicology, National Board of Forensic Medicine, Linköping, Sweden

Corresponding author:

Dr. Cristina Rodríguez-Antona, Spanish National Cancer Research Center (CNIO), Madrid, Spain. Ph. +34 917 328 000; Fax. +34 912 246 972; crodriguez@cnio.es

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Translational Relevance

Paclitaxel treatment frequently cause peripheral neuropathy, an adverse event that can limit treatment course and lead to permanent symptoms drastically decreasing quality of life. Our group has contributed to the identification and validation of common polymorphisms in *EPHA* genes associated with paclitaxel neuropathy, but a large part of the inter-individual variation in neuropathy remains unexplained. We hypothesized that low-frequency variants with strong effects may contribute to the neuropathy variability in patients. By performing targeted exon sequencing of candidate genes we found for the first time that patients carrying low-frequency non-synonymous coding variants in *EPHA5/6/8* contribute to paclitaxel-induced neuropathy susceptibility. Furthermore, these genes might also be relevant neuropathy markers for other neurotoxic drugs due to the involvement of Eph receptors in neuronal functions.

ABSTRACT

Purpose: Neuropathy is the dose limiting toxicity of paclitaxel and a major cause for decreased quality of life. Genetic factors have been shown to contribute to paclitaxel neuropathy susceptibility; however, the major causes for inter-individual differences remain unexplained. In this study we identified genetic markers associated with paclitaxel-induced neuropathy through massive sequencing of candidate genes.

Experimental Design: We sequenced the coding region of 4 *EPHA* genes, 5 genes involved in paclitaxel pharmacokinetics and 30 Charcot-Marie-Tooth genes, in 228 cancer patients with no/low neuropathy or high grade neuropathy during paclitaxel treatment. An independent validation series included 202 paclitaxel-treated patients. Variation-/ gene-based analyses were used to compare variant frequencies among neuropathy groups and Cox regression models were used to analyze neuropathy evolution along treatment.

Results: Gene-based analysis identified *EPHA6* as the gene most significantly associated with paclitaxel-induced neuropathy. Low frequency non-synonymous variants in *EPHA6* were present exclusively in patients with high neuropathy and all affected the ligand binding domain. Accumulated dose analysis in the discovery series showed a significantly higher neuropathy risk for *EPHA5/6/8* low-frequency non-synonymous variant carriers (HR=14.60, 95%CI=2.33-91.62, P=0.0042) and an independent cohort confirmed an increased neuropathy risk (HR=2.07, 95%CI=1.14-3.77, P=0.017). Combining the series gave an estimated 2.50-fold higher risk of neuropathy (95%CI=1.46-4.31; $P=9.1x10^{-4}$).

Conclusion: This first study sequencing *EPHA* genes revealed that low frequency variants in *EPHA6, EPHA5* and *EPHA8* contribute to the susceptibility to paclitaxel-induced neuropathy. Furthermore, EPHAs neuronal injury repair function suggests that these genes might constitute important neuropathy markers for many neurotoxic drugs.

INTRODUCTION

The anticancer agent paclitaxel is a microtubule inhibitor widely used in the treatment of many solid tumors (1). Peripheral neuropathy is its dose-limiting toxicity (2), and severe neuropathy cases with an important reduction in the quality of life of the patients are not rare (3, 4). The lack of effective treatments for the neuropathy creates an urgent need to identify markers that can help to personalize treatment and avoid severe neuropathy events. The patient genetic background has been proposed to play a relevant role in the susceptibility for suffering neuropathy (5). In this regard, paclitaxel pharmacokinetic (6, 7) and pharmacodynamic (8, 9) pathways have been included in studies of candidate genes and, more recently genome-wide association studies (GWAS) have been performed (10, 11).

Candidate gene studies, by us and other groups, have demonstrated that common variants in paclitaxel metabolizing enzymes and paclitaxel target (i.e. *CYP2C8*3* (12-14), *CYP3A4*22* (7), *TUBB2A* rs909964 and rs909965 (8, 9)) influence neuropathy risk, while genome wide genotyping has uncovered novel genes (10, 11). A GWAS by our group (11) suggested that the *EPHA* gene family, which plays a key role in the development of nervous system and in nerve injury repair (15-17), was a key player for paclitaxel neuropathy susceptibility. Meta-analysis of GWAS top hits showed that *EPHA5* rs7349683 reached genome-wide significance (11), and follow-up studies further supported that this variant (18), *EPHA6* rs301927 (9, 18) and *EPHA8* rs209709 (18) moderately increased paclitaxel-induced neuropathy risk. However, large part of the variation in paclitaxel-induced neuropathy remains unexplained.

Low-frequency variants with strong effects may contribute to the neuropathy variability observed in patients. To investigate this hypothesis sequencing technologies are required and, so far, only two exploratory studies following different strategies have been performed. In one we applied whole exome sequencing to few extreme neuropathy patients,

and identified defective *CYP3A4* variants associated with the neuropathy (19). The second study sequenced genes causative of familial polyneuropathies (Charcot-Marie-Tooth, CMT), and suggested *ARHGEF10* and *PRX* as chemotherapy-induced neuropathy markers (20). These initial studies are promising, however, the statistical power for a whole exome sequencing study is low and in the CMT analysis key genes were excluded.

Here, we performed targeted exome sequencing of genes with common variants associated with paclitaxel-induced neuropathy (*EPHA4, EPHA5, EPHA6* and *EPHA8*) plus genes involved in paclitaxel pharmacokinetics and in CMT. In total we sequenced 39 genes in 228 selected patients with high or no/low paclitaxel-induced neuropathy. The strongest association corresponded to *EPHA6*, and the relevance of low frequency *EPHA5/6/8* non-synonymous coding variants was validated in an independent cohort of 202 paclitaxel-treated patients. These results reveal *EPHA* genes as key players in chemotherapy-induced neuropathy and stress the importance of gene sequencing for identifying genetic risk factors of neuropathy.

PATIENTS AND METHODS

Patients

The discovery series was derived from a set of 449 breast or ovarian cancer patients treated with paclitaxel (97% in first line), with DNA available, no previous neurotoxic drug treatments and with clinical data and neuropathy assessment; some have already been reported (18, 19, 21). In these patients the neuropathy was homogenously graded (19), and 228 were selected for whole or targeted exon deep-sequencing, based on extreme-neuropathy phenotype. Among them, 131 were high-neuropathy patients that fulfilled the following criteria: grade 3 or 2 neuropathy (NCI-CTC v4) during paclitaxel treatment, no neuropathy risk factors (diabetes, alcoholism, AIDS or previous neuropathies), and treatment modifications due to neuropathy (dose reduction or treatment suspension) or neuropathy that lasted >6 months after paclitaxel treatment finished. The remaining 97 patients were no/low-neuropathy patients with no neuropathy signs or grade 1 neuropathy after receiving paclitaxel (Table 1).

The validation of results was performed in an independent series of 202 paclitaxel-treated patients with neuropathy data recorded cycle by cycle. Most patients had breast or ovarian tumors, 109 were Spanish (54%) and 93 Swedish (46%). 129 samples corresponded to a previous GWAS study (11), 37 to Spanish patients already described (18) and 36 samples were new cases collected in Spain. From all patients cumulative paclitaxel dose up to grade 2 (NCI_CTC v2/4) neuropathy was available (Table 1).

All individuals participating in the study were over 18 years of age, had been diagnosed of cancer with histological confirmation, a life expectancy of ≥12 weeks and ECOG performance status ≤2, adequate bone marrow and renal and hepatic function. 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.

Next generation sequencing (NGS)

From the 228 patients used in the discovery series, 196 samples were processed using the TruSeq Custom Amplicon Kit (Illumina) covering the coding plus 25 bp intronic flanking region of 39 genes that included: *EPHA4, EPHA5, EPHA6* and *EPHA8* (10, 11) plus additional genes involved in paclitaxel metabolism and transport (*ABCB1, CYP2C8*, *CYP3A4*, *SLCO1B1*, *SLCO1B3*) and a selection of 30 genes associated with CMT hereditary peripheral neuropathies (Fig. 1). Very conserved CMT genes with no/very few variants reported were not selected for sequencing (e.g. *ATL1, EGR2, GDAP1, GJB1, LMNA, PRPS1, RAB7A, YARS*). In brief, 150 ng of DNA extracted from peripheral blood (FlexiGene DNA Kit, Qiagen) was used to construct libraries and sequenced in a MiSeq sequencer (Illumina, Spain) with a paired-end mode using MiSeq Reagent Kit V3 (Illumina, Spain) and 600 cycles. In addition, whole exome sequencing was performed on the remaining 32 patients (16 with high neuropathy (8 have been reported (19)) and 16 patients with no neuropathy), as previously described (19). For the validation of the results, a TruSeq Custom Amplicon Kit (Illumina) including the coding and intronic flanking region of *EPHA5*, *EPHA6* and *EPHA8* was used.

Variant identification

Targeted NGS data was demultiplexed with MiSeq Reporter (Illumina). Alignment was performed using Smith-Waterman algorithm (22) using GRCh37/hg19 assembly as reference and Genome Analysis Toolkit v2 (GATK, (23)) was used for raw variant calling. For the 32 samples with whole exome sequencing data, alignment and variant calling were performed by RUbioSeq software v3.7 (24). In this software the alignment was performed using Burrows-Wheeler alignment (25), unmapped reads are realigned using BFAST (26) and for variant calling, GATK v2 was used (23). Variants were annotated with Snp Eff (http://snpeff.sourceforge.net/) and Variant Effect Predictor

(http://www.ensembl.org/info/docs/tools/vep/index.html), and only non-synonymous coding variants and those altering canonical splice sites, with P>0.001 for Hardy Weinberg Equilibrium were considered in subsequent steps. Supplementary Table 1 indicates gene and transcript references.

Variants included in the analysis were: i) those previously described in public databases (dbSNP, http://www.ncbi.nlm.nih.gov/SNP/; Exome Aggregation Consortium (ExAC), http://exac.broadinstitute.org), and ii) variants not previously described with: high variant call quality (Q>30), read depth >10X and alternative variant frequency higher than 0.3 in at least one individual. Sequencing artefacts, defined as nucleotide changes detected in >20 samples in the sequencing panel but never described in ExAC, were omitted from the analysis. We defined loss of function (LOF) variants as those introducing stop codons (nonsense), variants disrupting canonical splice sites and indels disrupting the reading frame. Template and configuration files for alignment and scripts are available at https://github.com/htejero/PaclitaxelNeuropathy.

Validation of variants was performed by Sanger sequencing with an ABI PRISM 3700 DNA Analyzer capillary sequencer (Applied Biosystems) on 3% of the LOF and missense variants included in the analysis.

Data analysis

Variants were classified as "common variants" if they had a minor allele frequency (MAF) ≥0.5% in the more than 30.000 sequenced non-Finnish Europeans from ExAC. Variants were classified as "low frequency variants" if they had a MAF <0.5% in the non-Finnish Europeans from ExAC and MAF <1% in 578 Spanish exomes from the CIBERER Spanish Variant Server (http://csvs.babelomics.org/). The purpose of including the Spanish data was to detect population specific variants, because of the small sample size (n<600) the

MAF threshold in this population was less stringent. For common variants, the frequency of each variant in the high versus no/low neuropathy group was compared with a Chi^2 or Fisher test. For low frequency variants, the association with paclitaxel-induced neuropathy was assessed with the gene-based Burden test (27) using the SKAT package and R statistical software (http://www.R-project.org/). Scripts are available at https://github.com/htejero/PaclitaxelNeuropathy. Based on statistical power calculations, only genes with ≥ four rare variants were included in the analysis.

The study followed a 2-step design in which the best candidates from the discovery phase were selected for validation in an independent cohort of paclitaxel-treated patients (Table 1 shows discovery and validation series). No correction for multiple testing was performed. For samples with cycle by cycle neuropathy data, the association between *EPHA* variants and paclitaxel neuropathy risk was tested using Kaplan-Meier analysis, modelling the cumulative dose of paclitaxel up to the development of neurotoxicity grade ≥2. Patients with no or low neuropathy (grade 0/1) were censored at total administered cumulative dose. We also evaluated the association using univariate and multivariable Cox regression analysis (14). Country of origin and treatment schedule (1h versus 3h infusion) were included as covariates in the multivariate analyses. SPSS software package v.19 was used for these analyses. P values less than 0.05 were considered statistically significant.

RESULTS

Study population and NGS

NGS was performed on selected cases: 131 patients with high neuropathy (grades 2/3 that lasted a mean of 55 months) despite low accumulated paclitaxel dose (median= 1295 mg) and 97 patients with no/low neuropathy (grades 0/1) despite high accumulated paclitaxel dose (median= 1485 mg) (Table 1). In addition, 33% of patients in the high neuropathy group had paclitaxel dose reductions or treatment suspensions caused by the neuropathy.

Sequencing of 39 candidate genes in the 228 patients identified 277 coding non-synonymous or canonical splice site variants (266 missense, 3 in-frame deletions, 8 LOF; Suppl. Table 1). From these, 86 were common variants and 191 low-frequency variants.

At least one common variant was identified in each sequenced gene, except for *CYP3A4*, *EPHA4*, *HSPB1*, *HSPB8*, *NEFL*, *NDRG1* and *SPTLC2*. When the presence of these common variants was compared among the neuropathy groups, association with paclitaxel neuropathy was found for only 2 SNPs located in *CYP2C8* and *PRX* ($P<0.05$; Suppl. Table 2).

The 191 low frequency variants were distributed among all sequenced genes, except for *NEFL* and *NGF*. Of these 191 variants, 8 were loss of function (3 altered canonical splice sites, 2 were nonsense variants and 3 were indels causing frameshifts leading to premature stop codons; Table 2).

Gene-based analysis of paclitaxel-induced neuropathy in the discovery series

Analysis of the low frequency variants identified *EPHA6* as the gene most significantly associated with paclitaxel-induced neuropathy (Table 3). The 5 carriers of these variants were all high neuropathy patients with an amino acid change in the ephrin receptor ligand binding domain of the protein. Remarkably, no *EPHA6* variant carriers were present in the no/low-neuropathy group, suggesting a strong effect on neuropathy. One additional gene

had this characteristic (*SEPT9*), but results did not reach statistical significance level. The other two *EPHA* genes analyzed, *EPHA5* and *EPHA8*, have a similar biological function as *EPHA6* (15-17) and also belonged to the high-neuropathy risk group of genes (Table 3). In *EPHA5*, 5 carriers had high neuropathy versus 1 with low neuropathy; and in *EPHA8,* 9 carriers were in the high neuropathy and 6 in the no/low neuropathy group (Fig. 2; Suppl. Table 1). The highly conserved *EPHA4,* with only 2 carriers, one in each group, could not be analyzed.

Some of the discovery series patients had cycle by cycle neuropathy data available and among these, 3 were carriers of low-frequency variants in *EPHA5/6/8* genes (one variant in each gene). Accumulated paclitaxel dose analysis revealed that these patients had a significantly higher risk to suffer from neuropathy than patients without *EPHA* low frequency variants (HR=14.60, 95%CI=2.33-91.62, P=0.0042; Fig. 3A).

Low frequency variants in *EPHA6, EPHA5* and *EPHA8* confirmed as neuropathy risk factor in the validation series

Sequencing *EPHA5/6/8* in an independent cohort of 202 patients treated with paclitaxel and detailed cycle by cycle neuropathy data (Table 1), revealed 15 carriers of low frequency missense variants in these genes (one corresponded to *EPHA6,* one to *EPHA5* and 13 to *EPHA8*). These variants were combined and an accumulated paclitaxel dose analysis revealed that low frequency *EPHA5/6/8* variants conferred increased risk of neuropathy (HR=2.07, 95%CI=1.14-3.77, P=0.017; Fig. 3B).

Combining discovery and validation series, resulted in a HR of 2.50 (95%CI=1.46-4.31) with a P value of 9.1×10^{-4} (Fig. 3C).

DISCUSSION

Paclitaxel induced-neuropathy is a clinically relevant toxicity affecting large number of cancer patients. Genetic variation has been shown to influence susceptibility to paclitaxel-induced neuropathy, however, a large part of the variation remains unexplained. Low-frequency variants with strong effects may explain part of the variability. To investigate this hypothesis, we performed massive sequencing of candidate genes in patients selected based on extreme-neuropathy phenotype. Gene-based analysis identified, for the first time, low frequency genetic variants in *EPHA5/6/8* as risk factors of chemotherapy induced neuropathy. These results may provide a basis for personalizing paclitaxel treatment and decreasing the incidence of severe chemotherapy-induced neuropathies.

GWAS studies have identified common variants in *EPHA* genes with moderate effects on paclitaxel-induced neuropathy (E*PHA5*-rs7349683, *EPHA6*-rs301927, *EPHA8*-rs209709 and *EPHA4*-rs17348202) (10, 11) and subsequent studies further supported the association of *EPHA5, EPHA6* and *EPHA8* polymorphisms (9, 18). Non-synonymous coding variants, potentially affecting protein function, are expected to have stronger effects on neuropathy than common regulatory variants (28). Following this idea, we performed a NGS study in *EPHA* genes, together with paclitaxel pharmacokinetics and hereditary peripheral neuropathy related genes. Gene-based analysis of our data revealed that low frequency missense variants in *EPHA6* increased paclitaxel-induced neuropathy risk. All these variants were located in the ephrin receptor ligand binding domain, suggesting an alteration of the protein function and further supporting the association. *EPHA5* and *EPHA8* followed a similar trend (Fig. 2). In total, 15% (19 of 131) of patients in the high neuropathy group carried low frequency non-synonymous coding variants in *EPHA5/6/8* genes. In the 202 patients of the validation series, 13 *EPHA8* variant carriers were identified but only one *EPHA6* and one *EPHA5* carriers were detected, suggesting that *EPHA6* and *EPHA5* variants (present in 5 out of the 131 patients

with high-neuropathy of the discovery) are less frequent in an unselected patient population, including many moderate-neuropathy patients (not represented in the discovery set). Thus, *EPHA6* and *EPHA5* variant carriers were scarce in the validation series, and the calculated EPHA-effect mainly derived from *EPHA8*. Despite this, the accumulated dose analysis is a sensitive approach (18, 21) and was able to detect a statistically significant association. Altogether, these data suggest a relevant role for *EPHA5/6/8* genes in paclitaxel-induced neuropathy and indicates a high impact of low frequency variants missed in GWAS.

Eph receptors are tyrosine kinases involved in neural development (15) and nerve regeneration after damage (17, 29) among other functions: EphA4 controls axon sprouting/ nerve regeneration after spinal cord injury (30-32); EphA5 plays an important role in the initiation of the early phases of synaptogenesis (33) and it has been found upregulated in mice with injured sciatic nerve (34); EphA6 is involved in neural circuits underlying aspects of learning and memory (35); and EphA8 induces neurite outgrowth through induction of sustained MAPK activity (36) while lack of this gene produces aberrant axonal projections (37). Knocking out EphA4, EphA5, Eph6 and EphA8 genes in mice, results in viable and fertile animals with different neurological phenotypes. EphA4 knockout mice have gross motor dysfunction (38-40) and altered axonal regeneration and functional recovery following spinal cord injury (41). Knocking-out the tyrosine kinase domain of EphA5 results in axon aberrations in topographic mapping and altered behavioral patterns (42, 43). EphA8 knockout mice have abnormal axonal projections in the spinal cord (37) and EphA6 knockout mice experienced behavioral deficits in learning and memory tests (35). Thus, these are crucial genes for neural development and nerve regeneration with a plausible link for the association found with paclitaxel-induced neuropathy.

In ExAC database 0.1% of the European non-Finish population are carriers of LOF variants in either *EPHA5, EPHA6* or *EPHA8,* and on >100,000 Islandic individuals, two

complete human knockouts for *EPHA5* and one for *EPHA6* were identified (44). So far, no phenotype has been assigned to these individuals who are apparently healthy subjects. However, based on the literature and on our results, a high susceptibility to drug-induced neuropathy would be expected.

Concerning other genes potentially associated with the neuropathy, in line with Beutler *et al* (20) we postulated that variants moderately affecting the function of CMT genes, while not being pathogenic, may increase the susceptibility to drug-induced neuropathy. We did not find low frequency variants in *PRX* and common variants in *ARHGEF10* associated with paclitaxel-induced neuropathy, although the 2nd and 3rd top protective genes were these two, similarly to Beutler *et al*. For the *ARHGEF10* common variant rs9657362 we also found a trend towards protection (20, 45). We also observed a trend towards increased neuropathy risk for other CMT genes (*SEPT9* and *SH3TC2*). Variability in results among studies may be related to differences in neuropathy definitions/ assessments, in tumor types and patient treatments, or in the distribution of low-frequency variants, which have shown to be population-specific. Thus, results need to be further explored and validated in large independent series.

With regards to the LOF variants detected in this study, three occurred in CMT genes (*ARHGEF10*, *IKBKAP* and *DHTKD1).* The patients with variants in *ARHGEF10* and *IKBKAP* belonged to the no/low neuropathy group, in agreement with the fact that activating rather than LOF mutations in *ARHGEF10* cause CMT (46) and that no phenotype is observed for *IKBKAP* heterozygous individuals (47). The variant in *DHTKD1* was present in two patients with different neuropathy, but recent data question the role of this gene in CMT disease (48, 49). Among the remaining LOF variants, two affected *EPHA* genes *(EPHA5* and *EPHA8)* and corresponded to high-neuropathy patients. One LOF variant occurred in the paclitaxel uptake transporter *SLCO1B1,* in a high neuropathy patient. Two occurred in

CYP3A4, a gene in which we have demonstrated that defective variants increased neuropathy risk (19). Two patients were carriers of the *CYP3A4*20* frameshift allele and belonged to the high-neuropathy group, but one patient with a splicing defect affecting the last exon belonged to the no/low neuropathy group. The effect of this latter variant on the splicing of the gene and how it affects function remains to be studied.

Although the main goal of this study was to identify neuropathy associated low-frequency coding variants, we also found two common polymorphisms associated with the neuropathy: *CYP2C8* rs1058930 (*CYP2C8*4*), for which previous studies have found contradictory results (9, 14), and *PRX* rs268674, which was associated with neuropathy risk here for the first time. Further studies should evaluate the relevance of these results.

Limitations of this study include gene selection, since relevant genes not yet connected with neuropathy susceptibility may have been excluded. There are also differences in the selection of patients in the discovery and validation series. In the discovery series, patients were mainly treated with paclitaxel as single agent whereas in the validation cohort, the majority of the patients were treated with paclitaxel in combination with carboplatin. No major differences in neuropathy development between paclitaxel/carboplatin therapy versus paclitaxel as single agent exist (50, 51). In addition, we adjusted the analysis using treatment schedule as covariate. Nevertheless, using a more homogenous series may have resulted in stronger association results. Detection of low/ moderate effects on neuropathy may require even larger samples sets, although the number of patients in this study is substantial and the neuropathy assessment was homogenously performed to reduce subjectivity (11, 19). On the whole, additional studies validating the results in extensive and well characterized series of patients, the development of a model integrating all different risk markers identified, and providing with a standardized methodology to perform the genetic testing would be required to implement these risk factors into the clinics.

In conclusion, this study proves a relevant role of *EPHA5*, *EPHA6* and *EPHA8* genes in paclitaxel-induced neuropathy susceptibility and suggests that sequencing studies, rather genotyping, would be adequate approaches to study genetic markers of neuropathy. Moreover, taking into account the role of these proteins in neural development and injury repair, *EPHA* variants may also confer increased neuropathy risk to many additional neurotoxic drugs. The final goal is to identify genetic risk factors that can help to personalize neurotoxic drug treatments and avoid severe chemotherapy-induced neuropathies that can seriously affect patients' quality of life.

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TABLES

Table 1. Characteristics of the patients in the discovery series (n=228) and validation series (n=202).

Characteristics	Discovery series		Validation series
	High neuropathy	No/ low neuropathy	Cycle by cycle neuropathy data
Number of patients	131	97	202
Age (years)			
Median (min-max)	54 (35-82)	48 (32-73)	60 (34-82)
Gender			
Female	131 (100%)	97 (100%)	187 (93%)
Male	0 (0%)	0 (0%)	15 (7%)
Tumor type			
Breast	121 (92%)	82 (85%)	47 (23%)
Ovary	10 (8%)	15 (15%)	120 (60%)
Others	0 (0%)	0 (0%)	35 (17%)
Type of paclitaxel treatment			
First line	129 (99%)	95 (98%)	192 (95%)
Second line[a]	2 (1%)	2 (2%)	10 (5%)
Paclitaxel treatment[b]			
FEC+T	81 (62%)	23 (24%)	0 (0%)
AC+T	18 (14%)	18 (19%)	35 (17%)
T+FEC	14 (11%)	29 (30%)	0 (0%)
C+T	10 (7%)	15 (15%)	156 (77%)
Others	8 (6%)	12 (12%)	11 (6%)
Number of paclitaxel cycles			
Median (min-max)	8 (3-13)	10 (6-27)	7 (2-44)
Paclitaxel accumulated total dose (mg)			
Median (min-max)	1295 (450-1600)	1485 (900-4059)	1225 (114-3150)
Maximum sensory neuropathy grade[c]			
Grade 0	0 (0%)	56 (58%)	32 (16%)
Grade 1	0 (0%)	41 (42%)	42 (21%)
Grade 2	30 (23%)	0 (0%)	78 (38%)
Grade 3	101 (77%)	0 (0%)	50 (25%)
Dose modifications due to neuropathy[d]			
Paclitaxel dose reduction	14 (11%)	0 (0%)	21 (10%)
Paclitaxel treatment suspension	29 (22%)	0 (0%)	23 (11%)

[a] Patients with second line paclitaxel treatment and no previous neurotoxic drugs in first line therapy.

[b] Some patients receiving chemotherapeutic drugs in combination with targeted therapy (bevacizumab, trastuzumab, denosumab or pertuzumab) are included in the table according to

the chemotherapy agents received. 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. AC+T: doxorubicin 60mg/m^2 and cyclophosphamide 600 mg/m^2, every 21 days, followed by paclitaxel 80mg/m^2, every 7 days. T+FEC: paclitaxel 80 mg/m^2, every 7 days, followed by 5-fluorouracil 600 mg/m^2, epirubicin 90 mg/m^2 and cyclophosphamide 600 mg/m2, every 21 days. C+T: carboplatin AUC5-6 and paclitaxel 175mg/m^2, every 21 days.

[c] NCI-CTC v2/4.

[d] When in the same patient paclitaxel dose was first reduced and later on paclitaxel treatment was suspended, the patient is included in the table as “treatment suspension”.

Table 2. Loss of function variants in the discovery series.

Gene	Type of gene	Variant[a]	Protein change	Nr individuals, Status	Discovery series group	Variant ID[b]	ExAC browser MAF[c]
ARHGEF10	CMT	c.1521_1522delAT[c]	p.Ala509His fs*515	1, Heterozygous	No/low NP	rs765378810	0.000066
IKBKAP		c.150+1G>A[c]	Splicing defect	1, Heterozygous	No/low NP	-	-
DHTKD1		c.1160-1G>C[c]	Splicing defect	2, Heterozygous	Both	rs760767010	0.000017
EPHA5	GWAS	c.2722dupT	p.Tyr908Leu fs*921	1, Heterozygous	High NP	-	-
EPHA8		c.1822C>T	p.Gln608*	1, Heterozygous	High NP	-	-
CYP3A4	PK	c.1461_1462insA (*CYP3A4*20*)	p.Pro488Thr fs*494	2, Heterozygous	High NP	rs67666821	0.00028
CYP3A4		c.1417-1G>C	Splicing defect	1, Heterozygous	No/low NP	rs141749477	0.0000083
SLCO1B1		c.1738C>T	p.Arg580*	1, Heterozygous	High NP	rs71581941	0.0016

[a] Genomic position and reference transcript are indicated in Supplemental Table 1.
[b] Variants not present in ExAC browser are indicated by "-".
[c] Variants not present in CMT databases (Inherited Peripheral Neuropathies Mutation Database http://www.molgen.vib-ua.be/CMTMutations/Mutations/MutByGene.cfm and OMIM http://www.omim.org/).
CMT: Charcot-Marie-Tooth; GWAS: Genome Wide Association Study; PK: pharmacokinetics; NP: neuropathy; MAF: minor allele frequency.

Table 3. Genes associated with paclitaxel-induced neuropathy using the gene-based burden test in the discovery series.

Gene	P-value	Number of variants carriers (variants)[a]	
		High neuropathy group, n=131	No/ low neuropathy group, n=97
Neuropathy risk			
EPHA6	0.041	5 (T72A,N127H,R162T,V196L)	0
SEPT9	0.072	4 (S96L,T235I,D348N,R355W)	0
SH3TC2	0.081	14 (T27A,V230A,T366A,S433L,Y510S,A590T,R658H,H696R,T755I,S831N,T1098P,D1229V)	4 (V230A,P251S,T1098P,D1229V)
EPHA5	0.219	5 (A49S,R494C,A611T,E678V,Y908fs)	1 (R238Q)
DHTKD1	0.271	9 (E42G,N107I,S114P,Q138K,A210S,c.1160-1G>C,T461K,I762del)	3 (I386V,c.1160-1G>C,G729R)
MFN2	0.323	6 (N63H,G298R,T423A,R468H,R663C)	2 (R468H,R707W)
LRSAM1	0.596	6 (I228M,F253V,Q409E,L500F,Q573K,L639P)	3 (S183L,R594C,Q697R)
SLCO1B3	0.737	5 (R23C,S64T,N145S,V235M)	3 (F36L,N145S,T414I)
ABCB1	0.752	5 (N183S,I261V,K624R,V835L)	3 (I261V,S1141T,R1225P)
EPHA8	0.785	9 (P321L,V365M,V444M,E462G,E464G,L559F,Q608*,A791V,D940H)	6 (G160S,I360V,V365M,E462G,Q525R,R679Q)
SBF2	0.787	7 (E304K,P339L,S730A,G775S,R890G,E1401K,K1672del)	3 (D289E,T1253S,A1849V)
SLCO1B1	0.800	4 (T10I,L193I,R580*,I656V)	3 (L193I,G210V)
Neuropathy protection			
TRPV4	0.082	1 (A293D)	4 (R160Q,R391W,T504A,S824L)
PRX	0.138	3 (M670V,P756L,D1013N)	6 (M670V,S751P K1062N,G1257R,E1360del,E1394D)
ARHGEF10	0.154	4 (S688N,H733Y,T811N,H1197Y)	7 (A509Hfs,S688N,H733Y,H834R,P956L,A960P)
NTRK1	0.261	2 (L79Q,G192A)	4 (L247P,Q570R,G714S,A779G)
SCN9A	0.456	4 (K40E,K655R,V1428I,L1916F)	5 (P74H,T152N,K655R,D1219E,L1267V)
IKBKAP	0.571	3 (M182K,R629H,G1013S)	4 (c.150+1G>A,M182K,S339R,R629H)
GARS	0.654	4 (C41R,R101H,S470F,T587M)	5 (T268I)
FAM134B	0.701	3 (P6L,V156F,S382T)	4 (M185V,V203M,Q379E,S382T)
Equal risk and protection			
AARS	0.650	5 (P234S,G275D,I579M)	5 (K81E,P234S,G275D,I579M)
FIG4	0.693	3 (I41T,K278N)	3 (I51V,A397P,E734K)
FGD4	0.712	3 (T79I,S392T,V717M)	3 (R275Q,V461A,D521G)
CYP3A4	0.795	4 (T185S,P389S,P488fs)	4 (R130Q,R162Q,T363M,c.1417-1G>C)

[a] Genomic position and reference transcript are indicated in Supplemental Table 1.

FIGURE LEGENDS

Figure 1. Genes selected for targeted NGS.

The NGS panel included 39 genes classified into two categories: 1) four *EPHA* genes involved in neural processes and found to be associated with taxane-induced neuropathy through GWAS; 2) 35 additional genes selected for an exploratory study, involved in paclitaxel pharmacokinetic (PK) or causative of Charcot-Marie-Tooth. Variants previously described to be associated with paclitaxel-induced neuropathy are included in the graph and the corresponding references provided.

Figure 2. Non synonymous *EPHA* coding variants in the discovery series.

The low frequency variants in *EPHA6*, *EPHA5* and *EPHA8* are represented along the protein sequences. In red variants in the high neuropathy group; in green variants in the no/low neuropathy group of patients. Protein domains are depicted according to Pfam database. Illustrator for Biological Sequences was used to create the graphs (http://ibs.biocuckoo.org/).

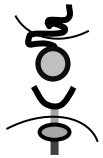
Figure 3. Kaplan-Meier analysis of paclitaxel-induced neuropathy.

Patients were grouped according to the absence (Without) or presence (With) of low-frequency variants in *EPHA5*, *EPHA6*, and *EPHA8,* and the cumulative dose of paclitaxel up to the development of grade 2 peripheral sensory neuropathy was compared. A) Discovery series (n=25). B) Validation series (n=202). C) Analysis combining patients from discovery and validation series (n=227). P values correspond to multivariable Cox regression analyses including country of origin and treatment schedule as covariates.

Figure 1

1) GWAS nerve repair

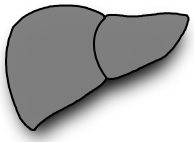
EphA receptors



Gene	(variant [ref])
EPHA4	(rs17348202 [11])
EPHA5	(rs7349683 [10,11,18])
EPHA6	(rs301927 [9,11,18])
EPHA8	(rs209709 [11,18])

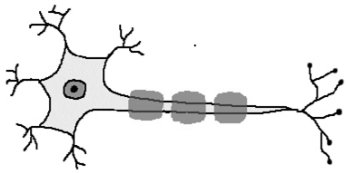
2) Exploratory study

Paclitaxel PK



Gene	(variant [ref])
CYP2C8	(**3* rs11572080 [12-14])
CYP3A4	(**20* rs67666821 [19]; **22* rs35599367 [7])
ABCB1	
SLCO1B1	
SLCO1B3	

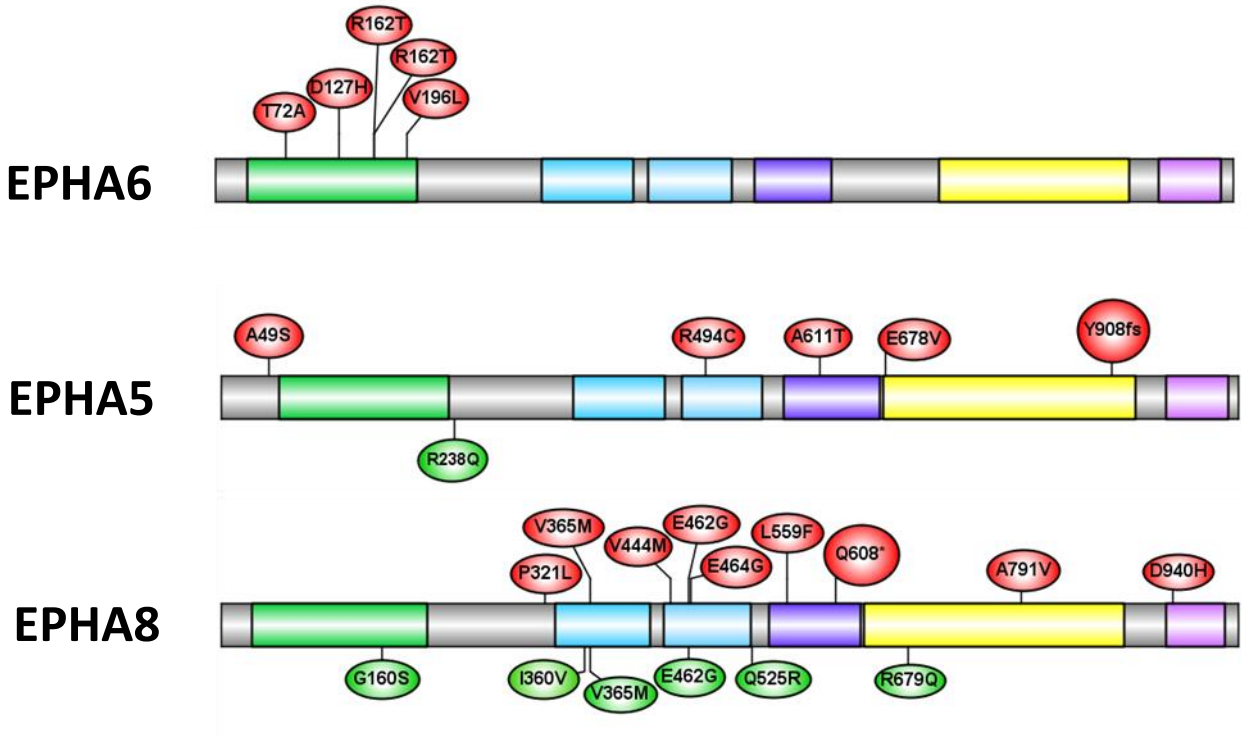
Charcot Marie Tooth genes



Gene	(ref)				
AARS	*FGD4*	*IKBKAP*	*MFN2*	*NTRK1*	*SEPT9*
ARHGEF10 [20]*	*FIG4*	*KIF1B*	*MTMR2*	*PMP22*	*SH3TC2*
CCT5	*GARS*	*LITAF*	*NDRG1*	*PRX* [20#]	*SPTLC1*
DHTKD1	*HSPB1*	*LRSAM1*	*NEFL*	*SBF2*	*SPTLC2*
FAM134B	*HSPB8*	*MED25*	*NGF*	*SCN9A*	*TRPV4*

*rs9657362, rs2294039 & rs17683288. # *PRX* rare variants

Figure 2



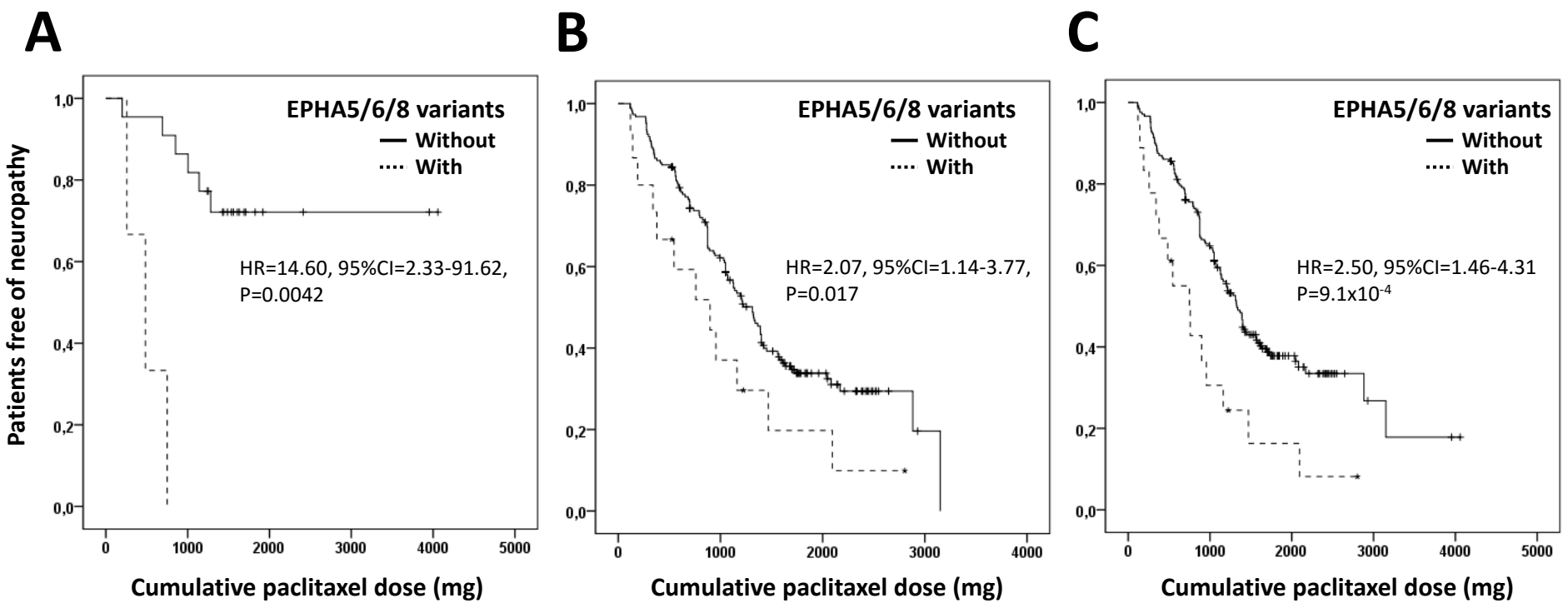
Neuropathy group

- High neuropathy
- No/ low neuropathy

Protein domains

- Ephrin receptor LB domain
- Fibronectin type III domain
- EphA2 TM domain
- Protein tyrosine kinase
- SAM domain

Figure 3


A
B
C
Patients free of neuropathy
EPHA5/6/8 variants
Without
With
HR=14.60, 95%CI=2.33-91.62,
P=0.0042
HR=2.07, 95%CI=1.14-3.77,
P=0.017
HR=2.50, 95%CI=1.46-4.31
P=9.1x10-4
1,0
0,8
0,6
0,4
0,2
0,0
0
1000
2000
3000
4000
5000
Cumulative paclitaxel dose (mg)