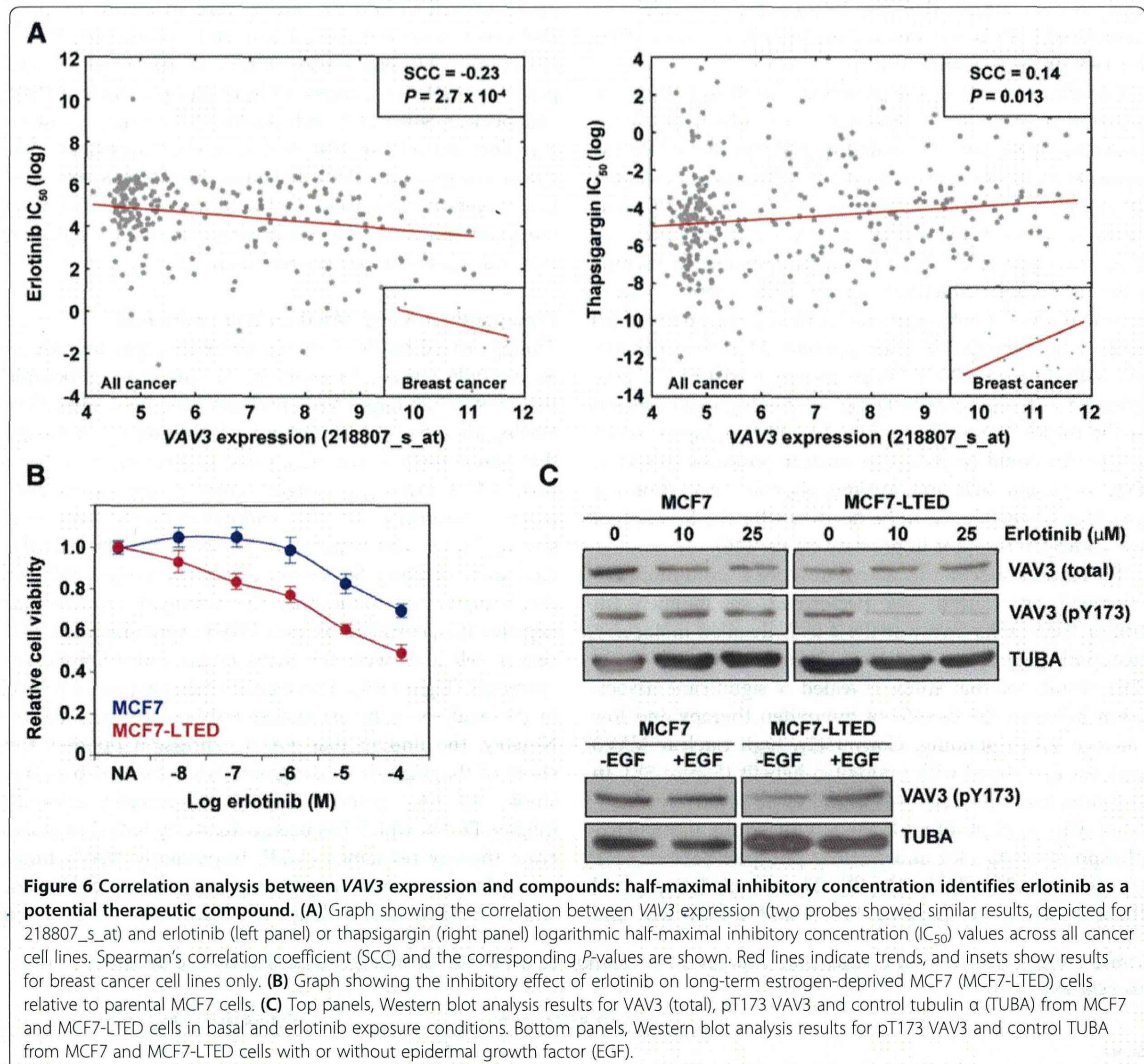

Figure 6 Correlation analysis between *VAV3* expression and compounds: half-maximal inhibitory concentration identifies erlotinib as a potential therapeutic compound. (A) Graph showing the correlation between *VAV3* expression (two probes showed similar results, depicted for 218807_s_at) and erlotinib (left panel) or thapsigargin (right panel) logarithmic half-maximal inhibitory concentration (IC_{50}) values across all cancer cell lines. Spearman's correlation coefficient (SCC) and the corresponding *P*-values are shown. Red lines indicate trends, and insets show results for breast cancer cell lines only. **(B)** Graph showing the inhibitory effect of erlotinib on long-term estrogen-deprived MCF7 (MCF7-LTED) cells relative to parental MCF7 cells. **(C)** Top panels, Western blot analysis results for VAV3 (total), pT173 VAV3 and control tubulin α (TUBA) from MCF7 and MCF7-LTED cells in basal and erlotinib exposure conditions. Bottom panels, Western blot analysis results for pT173 VAV3 and control TUBA from MCF7 and MCF7-LTED cells with or without epidermal growth factor (EGF).

observations, exposure to erlotinib significantly reduced the viability of MCF7-LTED relative to MCF7 cells (Figure 6B). VAV3 expression was not reduced by exposure to erlotinib (contrary to exposure to YC-1), but we observed a partial reduction in pY173 VAV3 in MCF7-LTED cells (Figure 6C, top panels). Accordingly, exposure to EGF increased pY173 VAV3 in this setting (Figure 6C, bottom panels). Collectively, these results further endorse a critical role for VAV3 in endocrine therapy resistance.

Discussion

The results of this study suggest that VAV3 function mediates the response to endocrine therapies in breast cancer and, as a result, the acquisition of resistance. In this context, VAV3 might be a key effector whose expression is differentially regulated by ERα [7]. Thus, the expression regulation of VAV3 would be relatively more dependent on ERα in the endocrine therapy–resistant setting. Conversely, in previous studies, researchers have proposed that VAV3 is an activator of ERα [55,56]. These observations could indicate the existence of a feedback mechanism that would ultimately regulate growth factor signaling. Indeed, VAV3 has been shown to activate receptor protein tyrosine kinases and RAC1 [54-56], and an inhibitor of this protein can decrease both estrogen-induced cell proliferation and MCF7-tamoxifen-resistant cell growth [56]. Notably, authors of an independent report identified VAV3 as a marker for posttreatment recurrence of prostate cancer [57]. Together with our analysis of VAV3 in breast tumors, these observations further endorse the link

between the VAV3-RAC1-PAK1 signaling axis and resistance to endocrine therapies. Nevertheless, analysis of differential gene expression by exposure to YC-1 may point to complementary mediators of endocrine therapy resistance. Activation of ERBB4 has previously been linked to this setting [58-60], and two other identified perturbations (*GLI3* and *PTCH1*) belong to the Hedgehog signaling pathway, which has been highlighted as a possible therapeutic target in this setting [61]. Whether these proteins act functionally in concert with VAV3 or whether they represent necessary alterations in different biological processes or pathways remains to be determined.

The association between genetic variation in *VAV3* and the response to tamoxifen could allow the stratification of patients according to potential clinical benefit. However, this association should be replicated in independent studies with larger samples. The rs10494071 minor allele has a relatively high frequency in the Japanese population, but is rare in individuals of European ancestry (45% and 5%, respectively, according to HapMap data). This is also the case with a variant in linkage disequilibrium with rs10494071 (data not shown). These observations indicate that an attempt to replicate the association in a non-Japanese population will require dense genotyping at the specific locus.

Although the results of the genetic association should be replicated, they are consistent with the anticipated functional role of VAV3 and with the observations made in gene expression analyses. In our present study, we identified an association between the rs10494071 minor allele and better tamoxifen response, and, in turn, we found in our analysis of a tumor data set that low *VAV3* expression correlates with better tamoxifen response [45]. Additionally, these observations seem to be coherent with the role of the rs10494071 variant as an expression quantitative trait locus for *VAV3*, with the minor allele being associated with significantly lower gene expression in monocytes [44]. Importantly, in a previous study in which the researchers identified VAV3 as a marker for posttreatment recurrence of prostate cancer, the association was in the same direction [57]. Moreover, these results are consistent with, and the conclusions further endorsed by, the associations revealed for nuclear VAV3 and tamoxifen therapy response, as well as the observed correlations between the expression of VAV3 and known tumor markers linked to therapy response. However, further work is required to elucidate the functional difference between nuclear and cytoplasmic VAV3, which is reminiscent of the results for PAK1 [49] and could be linked to the activation of the androgen receptor, as previously shown in prostate cancer [46,62].

It has been firmly established that growth factor signaling influences the response to endocrine therapies and, consequently, the acquisition of resistance. Among other evidence, overexpression of growth factor receptors, including EGFR, has been associated with decreased sensitivity to endocrine therapy and poorer prognosis [63]. Akin to this observation, other researchers have reported that cell models of endocrine therapy resistance overexpress several growth factor receptors, also including EGFR [17]. In turn, these observations have led to the design of clinical trials to assess the target inhibition of the receptors [64]. In this scenario, the analysis of VAV3 expression and/or function could potentially help to identify patients that may benefit from therapies aimed at preventing and/or overcoming endocrine therapy resistance.

Conclusions

In this study, we have identified VAV3 as a critical mediator of endocrine therapy resistance in breast cancer downstream of ERα and growth factor receptor signaling. The expression of VAV3 may be specifically regulated by ERα in the endocrine therapy–resistant setting. The results of our genetic and immunohistochemical studies indicate that *VAV3*/VAV3 represents a promising biomarker for predicting the response to endocrine therapies. Despite the lack of targeted therapies for VAV proteins, inhibition of EGFR signaling could potentially prevent and/or overcome endocrine therapy resistance mediated by VAV3.

Additional files

Additional file 1: Table S1. Results from the chemical compound screen.

Additional file 2: Table S2. Values of YC-1 IC_{50} (μM) in breast cancer cell lines.

Additional file 3: Figure S1. Assessment of the activation of sGC in the viability inhibition of MCF7-LTED cells. **(A)** BAY 41-2272 shows an effect, but less than that of YC-1. **(B)** A-350619 (activator of sGC) and sulindac sulfide (inhibitor of phosphodiesterase) do not show the predicted effects in MCF7-LTED cells. In fact, the contrary is observed; A-350619 appears to be more effective in MCF7 cells.

Additional file 4: Figure S2. Study of the binding mode of YC-1 to ERα. **(A)** Predicted binding mode of YC-1 (purple) in the unconstrained conformation of ERα (chain C, PDB code 3OS8). The binding mode of WAY6 (white sticks) is shown as reference. **(B)** Docking pose of YC-1 (purple) in the unconstrained conformation of ERα (chain C, PDB code 3OS8) resembling the experimentally observed structure. This binding mode is three score units worse than the one shown above. The binding mode of WAY6 (white sticks) is shown as reference.

Additional file 5: Figure S3. Signaling pathways differentially expressed between breast cancer cell lines "sensitive" and "insensitive" to YC-1 exposure (defined by the IC_{50} 10 μM threshold). **(A)** High expression of the cell cycle pathway shows significant association (false discovery rate <5%) with YC-1 sensitivity. Pathway annotations correspond to those in the Kyoto Encyclopedia of Genes and Genomes (KEGG). **(B)** High expression of the ribosome pathway shows significant association with lower YC-1 sensitivity.

Additional file 6: Table S3. Pathways potentially associated (false discovery rate <5%) with the breast cancer response to YC-1.

Additional file 7: Figure S4. Analysis of ERα localization and levels following exposure to YC-1. **(A)** ERα is mislocalized upon exposure to YC-1 in both MCF7 and MCF7-LTED cells. **(B)** Total ERα levels are reduced upon exposure to YC-1 in both MCF7 and MCF7-LTED cells, although relatively more in MCF7-LTED cells. **(C)** Subcellular fractionation does not

reveal differences for ERα. Ponceau protein staining and detection of the 62 kDa nucleoporin (NUP62) were used as loading controls.

Additional file 8: Figure S5. Expression analysis with exposure to YC-1. **(A)** High expression of the Ribosome pathway (false discover rate <5%) is shown in the parental MCF7. **(B)** Top panels, the Ribosome pathway is significantly altered (that is, underexpressed) in MCF7 cells, but not in MCF7-LTED cells, exposed to YC-1. Bottom panels, both MCF7 and MCF7-LTED cells show underexpression of the cell cycle pathway with exposure to YC-1. **(C)** Western blot analysis results of phospho-serine 235/236 S6 ribosomal protein, E2F1 and control TUBA in MCF7 and MCF7-LTED cells in basal or YC-1-exposed conditions.

Additional file 9: Table S4. Pathways differentially expressed (false discovery rate <5%) in MCF7 and/or MCF7-LTED cells, in basal and/or YC-1 conditions.

Additional file 10: Table S5. Differential expression analysis of predicted E2F1 target sets (false discovery rate <1%) in MCF7 and MCF7-LTED cells exposed to YC-1.

Additional file 11: Figure S6. Results from RAC1 activity assays with depletion and/or reconstitution of MYC-Vav3. Left panel, graph depicting RAC1 activity from triplicate assays in the conditions depicted across the *x*-axis. The asterisks correspond to significant differences ($P < 0.05$). Right panels, Western blot analysis results of total VAV3, MYC (for MYC-Vav3) and control TUBA in MCF7 and MCF7-LTED cells transduced with shRNA control (pLKO.1) or shRNA-*VAV3* plus MYC-Vav3 constructs.

Additional file 12: Table S6. Results of the GWAS and the replication study for SNPs in *VAV3*.

Abbreviations

ChIP: Chromatin immunoprecipitation; EGFR: Epidermal growth factor receptor; ERα: Estrogen receptor α; GSEA: Gene set expression analysis; GWAS: Genome-wide association study; IC_{50}: Half-maximal inhibitory concentration; LTED: Long-term estrogen-deprived; MTT: Methylthiazol tetrazolium; PDB: Protein Data Bank; sGC: Soluble guanylyl cyclase; shRNA: Short hairpin RNA; SNP: Single-nucleotide polymorphism.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HA, AU and MAP conceived the project and coordinated the experiments and data analyses. HA, PH and RLB performed the compound screen. JSM, NB and MAP carried out the microarray data analyses. XB performed the protein structure analyses. AI, EN and WZ performed the ChIP data analysis. LC, HA, MAP and LDC performed the targeted ChIP assays. HA, NG, GM and LGB performed the cellular and molecular studies. HA and LC performed the *ESR1* shRNA-based assays. KK, TM, YN and HZ performed the genetic association study. NG, FC, MTS, ARV, MG, AIE, ABRP and XRB performed the tumor and immunohistochemical studies. JBo, EK, GPT, TF, DCS and OS performed the analyses of the Swedish breast cancer study. HA, JSM, MV, ME and MAP contributed the cell lines and performed the erlotinib analysis. RGM, MPHMJ, JBr, AF, JBa, RC, KLB, KEC, JAK and AV contributed the reagents and to the experimental design. MAP drafted the manuscript. All authors read and approved the final manuscript.

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Author details

[1]Breast Cancer and Systems Biology Unit, Translational Research Laboratory, Catalan Institute of Oncology (ICO), Bellvitge Institute for Biomedical Research (IDIBELL), Avda. Gran via 199, L'Hospitalet del Llobregat, Barcelona 08908, Catalonia, Spain. [2]Division of Molecular Carcinogenesis, Center for Biomedical Genetics and Cancer Genomics Centre, The Netherlands Cancer Institute, Plesmanlaan 121, Amsterdam 1066 CX, The Netherlands. [3]Center for Genomic Medicine, RIKEN, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama City, Kanagawa 230-0045, Japan. [4]Department of Physical Chemistry, Institute of Biomedicine (IBUB), Avda. Diagonal 643, University of Barcelona, Barcelona 08028, Catalonia, Spain. [5]Catalan Institution for Research and Advanced Studies (ICREA), C/ Lluís Companys 23, Barcelona 08010, Catalonia, Spain. [6]ICO, Girona Biomedical Research Institute (IDIBGI), Hospital Josep Trueta, Avda. França s/n, Girona 17007, Catalonia, Spain. [7]Department of Genetic Engineering and Biotechnology, University of Dhaka, Dhaka 1000, Bangladesh. [8]Centre for Genomic Regulation (CRG), C/ Dr. Aiguader 88, Barcelona 08003, Catalonia, Spain. [9]Universitat Pompeu Fabra (UPF), C/ Dr. Aiguader 88, Barcelona 08003, Catalonia, Spain. [10]Department of Molecular Pathology, The Netherlands Cancer Institute, Plesmanlaan 121, Amsterdam 1066 CX, The Netherlands. [11]Department of Clinical and Experimental Medicine, Division of Oncology, Linköping University, County Council of Östergötland, Sandbäcksgatan 7, Linköping SE-58185, Sweden. [12]Department of Oncology, Karolinska University Hospital, Stockholm South General Hospital, Sjukhusbacken 10, Stockholm SE-11883, Sweden. [13]Department of Pathology, Molecular Pathology Research Unit, Massachusetts General Hospital, 13th St. Charlestown, Boston, MA 02129, USA. [14]Department of Cell Biology and Physiology, University of North Carolina at Chapel Hill, 111 Mason Farm Rd., Chapel Hill, NC 27599-7545, USA. [15]Department of Medical Oncology, Erasmus University Medical Center, Cancer Institute, PO Box 2040, Rotterdam 3000 CA, The Netherlands. [16]Translational Research Laboratory, ICO, IDIBELL, Avda. Gran via 199, L'Hospitalet del Llobregat, Barcelona 08908, Catalonia, Spain. [17]Department of Pathology, University Hospital of Bellvitge, IDIBELL, Avda. Feixa Llarga s/n, L'Hospitalet del Llobregat, Barcelona 08908, Catalonia, Spain. [18]Department of Medical Oncology, Breast Cancer Unit, ICO, IDIBELL, Avda. Gran via 199, L'Hospitalet del Llobregat, Barcelona 08908, Catalonia, Spain. [19]Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, 3970 Reservoir Rd., Washington, DC 20057, USA. [20]Department of Molecular and Cellular Pharmacology, University of Miami, Miller School of Medicine, 1600 NW 10th Ave., Miami, FL 33136, USA. [21]Department of Chemistry, University of Illinois, 505 South Mathews Ave., Urbana, IL 61801, USA. [22]Cancer Epigenetics and Biology Program (PEBC), IDIBELL, Avda. Gran via 199, L'Hospitalet del Llobregat, Barcelona 08908, Catalonia, Spain. [23]Department of Physiological Sciences II, School of Medicine, University of Barcelona, Avda. Feixa Llarga s/n, L'Hospitalet del Llobregat, Barcelona 08908, Catalonia, Spain. [24]Cancer Research Center (CSIC), University of Salamanca, Campus Miguel de Unamuno, Salamanca 37007, Spain. [25]Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. [26]Present address: Onkologikoa Foundation, Biodonostia, San Sebastián, Doctor Begiristain 121, Guipúzcoa 20014, Spain.

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Polygenic Inheritance of Paclitaxel-Induced Sensory Peripheral Neuropathy Driven by Axon Outgrowth Gene Sets in CALGB 40101 (Alliance)

Aparna Chhibber[1,3], **Joel Mefford**[2,3], **Eli A. Stahl**[4,5,6], **Sarah A. Pendergrass**[7], **R. Michael Baldwin**[1,3], **Kouros Owzar**[8], **Megan Li**[1,3], **Eric P. Winer**[9], **Clifford A. Hudis**[10], **Hitoshi Zembutsu**[11], **Michiaki Kubo**[12], **Yusuke Nakamura**[11,12,13], **Howard L. McLeod**[14], **Mark J. Ratain**[13], **Lawrence N. Shulman**[9], **Marylyn D. Ritchie**[7], **Robert M. Plenge**[4,5], **John S. Witte**[2,3], and **Deanna L. Kroetz**[1,3]

[1]Departments of Bioengineering and Therapeutic Sciences, University of California at San Francisco, San Francisco, CA

[2]Department of Epidemiology and Biostatistics, University of California at San Francisco, San Francisco, CA

[3]Institute for Human Genetics, University of California at San Francisco, San Francisco, CA

[4]Division of Rheumatology, Immunology, and Allergy, Division of Genetics, Brigham and Women's Hospital, Harvard Medical School, Boston, MA

[5]Medical and Population Genetics Program, Chemical Biology Program, Broad Institute, Cambridge, MA

[7]Center for Systems Genomics, Department of Biochemistry and Molecular Biology, The Pennsylvania State University, Eberly College of Science, The Huck Institutes of the Life Sciences, University Park, PA

[8]Alliance Statistics and Data Center, Duke University Medical Center, Durham, NC

[9]Dana-Farber Cancer Institute, Boston, MA

[10]Memorial Sloan-Kettering Cancer Center, New York, NY

[11]Laboratory of Molecular Medicine, University of Tokyo, Tokyo, Japan

[12]Center for Integrative Medical Sciences, Riken Center, Yokohama, Japan

[13]Department of Medicine, University of Chicago, Chicago, IL

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Corresponding Author: Deanna L. Kroetz, PhD, Department of Bioengineering and Therapeutic Sciences, University of California San Francisco, 1550 4th Street RH584E, San Francisco, CA 94158-2911, Phone: 415-476-1159, Fax: 415-514-4361, deanna.kroetz@ucsf.edu.
[6]Current address: Division of Psychiatric Genomics, Mt. Sinai School of Medicine, New York, NY

Conflict of Interest
The authors declare no conflict of interest.

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[14]Department of Pharmacotherapy and Experimental Therapeutics, University of North Carolina at Chapel Hill, Chapel Hill, NC

Abstract

Peripheral neuropathy is a common dose-limiting toxicity for patients treated with paclitaxel. For most individuals there are no known risk factors that predispose patients to the adverse event, and pathogenesis for paclitaxel-induced peripheral neuropathy is unknown. Determining whether there is a heritable component to paclitaxel induced peripheral neuropathy would be valuable in guiding clinical decisions and may provide insight into treatment of and mechanisms for the toxicity. Using genotype and patient information from the paclitaxel arm of CALGB 40101 (Alliance), a phase III clinical trial evaluating adjuvant therapies for breast cancer in women, we estimated the variance in maximum grade and dose at first instance of sensory peripheral neuropathy. Our results suggest that paclitaxel-induced neuropathy has a heritable component, driven in part by genes involved in axon outgrowth. Disruption of axon outgrowth may be one of the mechanisms by which paclitaxel treatment results in sensory peripheral neuropathy in susceptible patients.

Keywords

paclitaxel; neuropathy; polygenic; heritability; pathway

Introduction

Peripheral neuropathy is a common and often dose-limiting toxicity associated with cancer chemotherapy treatment. Paclitaxel is a chemotherapeutic agent in the taxane family, and functions by inhibiting microtubule assembly and inducing apoptosis. It is commonly prescribed in the treatment of carcinomas of the breast, ovary, lung, and head and neck[1]. Sensory peripheral neuropathy induced by paclitaxel is dose-dependent and is the most common toxicity associated with this microtubule inhibitor. Severe toxicity (Grade 3 or higher) generally occurs in 5–10% of patients although rates as high as 30% have been reported for certain dosage regimens[2]. Known risk factors for paclitaxel induced neuropathy include prior exposure to a neurotoxic agent or medical conditions associated with peripheral neuropathy, such as diabetes[2–6], though most patients who suffer from paclitaxel-induced neuropathy do not have an identifiable predisposition. The pathogenesis of paclitaxel induced peripheral neuropathy is unclear. Paclitaxel treatment may target axons, myelinating Schwann cells, or the dorsal root ganglion and neuron cell bodies of peripheral nerves[7]. At any of these sites, damage may be mediated by microtubule stabilization or mitochondrial disruption[8]. At very high single or cumulative doses almost all patients will experience some degree of peripheral neuropathy, but in certain susceptible patients neuropathy will occur at lower cumulative doses or with greater severity. Interindividual susceptibility to paclitaxel induced peripheral neuropathy may be driven by an overall increase in exposure to paclitaxel, or an increased sensitivity to damage or decreased capacity for repair at any of the putative targets of paclitaxel in the peripheral neuron.

Given the wide interindividual variability in incidence and severity of the toxicity independent of any known risk factors, it is likely that there is an underlying genetic basis

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for susceptibility to paclitaxel-induced neuropathy. Small candidate gene studies focusing on genes involved in paclitaxel pharmacokinetics and pharmacodynamics (*e.g.*, *ABCB1, CYP2C8*) or paclitaxel targets (*e.g.*, β-tubulin) have had mixed results, with some identifying variants associated with neuropathy[9–11], and others failing to replicate previous results[12, 13]. Recently, a genome-wide association study from this group[14] identified several SNPs with moderate effect size in *FZD3, FGD4*, and *EPHA5* associated with severity or dose at onset of paclitaxel-induced sensory peripheral neuropathy. An independent genome-wide study identified SNPs in *RWDD3* and *TECTA* associated with onset of paclitaxel-induced neuropathy[15], but these findings were not replicated by others[16]. The large number of putative causative variants identified, many with small effect size, and the discrepancies from study to study suggest a complex polygenic etiology for susceptibility to paclitaxel-induced neuropathy.

Pharmacogenomic studies, especially those involved in the study of drug toxicities, come with their own particular set of challenges. Sample sizes are often limited, and phenotype definitions can be imprecise[17]. This is compounded in cases where the toxicity does not appear to be driven by one or a few polymorphisms with large effect size, such as *CYP2D6* polymorphisms and morphine toxicity[18], but rather by a number of variants each with small potential contribution to disease, as we propose is the case for paclitaxel-induced peripheral neuropathy. For these phenotypes, determining the extent to which genetic variability contributes to a particular toxicity can be challenging. Traditional heritability studies require large numbers of siblings or family structures that are not practicable, especially when studying potentially toxic drugs. Even when evidence for a heritable component to toxicity is available, candidate gene/candidate variant studies or traditional genome-wide association studies will likely be unable to identify variants with small effects that together explain a large portion of the expected heritability.

Recently, a method has been developed to estimate additive genetic variation or narrow-sense heritability driven by common SNPs (i.e. those typically captured on genotyping platforms) in unrelated individuals using linear mixed models[19, 20]. This approach was applied to genome-wide SNP data in breast cancer patients treated with paclitaxel to determine the extent to which paclitaxel-induced sensory peripheral neuropathy is heritable and to identify causal SNPs driving this heritability.

Materials and Methods

Patient Data and Study Design

The patient cohort for this study was taken from the paclitaxel arm of CALGB 40101 (Alliance), a Phase III trial studying adjuvant therapy for patients with breast cancer; all patients in the current study were also enrolled in CALGB 60202 (Alliance), the pharmacogenomic companion study, and signed an IRB-approved, protocol-specific informed consent for use of their specimens. Paclitaxel was administered every two weeks over three hours at 175 mg/m^2 for four or six cycles. A total of 1,040 paclitaxel-treated individuals were included in the cohort; after quality control, including principal component analysis, call rate (>98%), and clustering performance, 859 Caucasian patients were retained for further analysis. Germline DNA was genotyped on the HumanHap610-Quad Genotyping

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BeadChip (Illumina) platform. SNP quality control measures for minor allele frequency ($\geq$ 0.01), genotyping call rate ($\geq$99%), and Hardy-Weinberg equilibrium in controls (exact test $p \geq 0.001$) were applied using PLINK (v1.07). Genotyped data was imputed to call genotypes of un-typed SNPs using MACH[21, 22] (1.0) and the 1000 Genomes[23] Pilot I (June 2010) data from unrelated Caucasian (CEU) individuals as a reference; imputed data was filtered for $r^2 > 0.9$. Recent publications describe further details regarding the pharmacogenomic[14] and clinical[24] studies. Details regarding patient selection, SNP quality control and imputation are outlined in Supplemental Figure 1.

Phenotype

Two phenotypes are of interest in studying paclitaxel-induced neuropathy – severity of the neuropathy and cumulative dose at onset of neuropathy. These outcomes may be driven by distinct or overlapping sets of genes. Peripheral neuropathy was graded on a scale of 0 to 5 according to the National Cancer Institute Common Toxicity Criteria for Adverse Events (NCI-CTCAE) version 2.0. The distribution of neuropathy grades in our cohort (Figure 1) matches expected numbers from prior clinical trials[25, 26]. Because the linear mixed modeling approach requires a continuous quantitative or binary phenotype, both severity of neuropathy and dose at onset of neuropathy were treated as continuous variables. Severity of neuropathy was modeled using the highest grade of neuropathy over the course of treatment with log-transformed cumulative dose administered at highest grade of neuropathy (mg/m^2) as a covariate. For patients who did not experience the toxicity, cumulative dose administered over the course of the study was used as the covariate. Onset of neuropathy was modeled using deviance residuals from a time-to-event analysis as a continuous phenotype. The deviance residuals are a normalized transform of the martingale residuals, which estimate the difference at a particular cumulative dose t between observed (incidence of grade 2 or peripheral higher neuropathy, 0 or 1) and expected events (predicted hazard for neuropathy at dose t) for a given patient. Residuals from survival models have been previously used to model time to onset of various phenotypes as a quantitative trait when it is not possible to apply a survival model directly[27–29]. The time-to-event analysis was conducted using a null Cox proportional hazards model without predictors, with time defined as cumulative paclitaxel dose and event defined as first instance of grade 2 or higher peripheral neuropathy[14]. For patients who did not experience grade 2 or higher neuropathy, cumulative dose administered over the course of the study was used, producing right–censored dosage date. Deviance residuals from the Cox score test were calculated using the survival package in R[30, 31].

Pathway Definitions

Pathways evaluated were selected based on putative pathology for paclitaxel-induced neuropathy. Five Gene Ontology[32] (GO Release 2012-09-15) Biological Process terms were included: Axonogenesis (GO: 0007409), Myelination (GO: 0042552), Transmission of Nerve Impulse (GO: 0019226), Microtubule-Related Processes (GO: 0007017), and Mitochondrial Organization and Transport (GO: 0006839 and 0007005), along with a manually curated set of genes associated with congenital peripheral neuropathy[33] and a set of genes in the paclitaxel pharmacokinetic/pharmacodynamic pathway[34]. For GO terms, all possible genes (regardless of evidence code) were included. For each pathway, gene

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boundaries for the largest isoform of each gene were extracted from the UCSC Table Browser using UCSC gene annotations from human genome build 37 (hg19). These gene boundaries (plus an additional 10 kb upstream and downstream) were used to extract all dbSNP135[35] SNPs in the gene regions. Pathway SNP lists were used to extract the pathway-specific portion of the genome in PLINK (v1.07)[36].

For SNP sets grouped by position in the genome (genic vs. intergenic), gene and SNP annotations were extracted from the UCSC Table Browser using CCDS[37] gene annotations from human genome build 37 (hg19), and SNP annotations from dbSNP135. Genic regions were defined as 10 kb upstream and downstream of transcription start and stop sites. For genes with multiple CCDS isoforms, the longest isoform was used. The Biofilter[38] software (v2.0.0) was used to extract SNPs by genomic position.

Linear Mixed Modeling Heritability Analysis

Heritability estimates for the whole genome and for pathways were generated using the GCTA (v1.01) software tool[39]. We estimated the genetic relatedness matrix (GRM) for 859 Caucasians using all post-QC genotyped SNPs. Principal components analysis was conducted using GCTA, and the first 20 eigenvectors for each individual were used as covariates in all subsequent analyses to control for any remaining population stratification. To ensure that all subjects in the study were unrelated, we excluded one of each of a pair of individuals with genetic relationship greater than 0.03, roughly corresponding to second cousins or closer familial relationships; ten individuals were excluded in this step. An additional four individuals were excluded due to incomplete phenotype information for a final population of 845 unrelated Caucasians (Supplemental Figure 1). All analyses were restricted to autosomes, and were conducted with the assumption that causal SNPs will have the same allele frequency distribution as genotyped SNPs.

For pathway specific heritability analyses, a separate GRM was constructed for each pathway and for its complement (whole genome GRM excluding SNPs in the pathway) using the set of 845 unrelated Caucasians. Total genetic variance for severity and onset of neuropathy was partitioned simultaneously onto pathway and "non-pathway" SNPs. Likewise, for genomic position based heritability analyses, total genetic variance for both phenotypes was partitioned onto genic and intergenic regions. To correct for the simultaneous evaluation of multiple pathways, GCTA p-values were Bonferroni corrected by multiplying each p-value by the number of pathways tested together (seven in the first round and ten in the second round). Empirical distributions representing the null hypothesis that the trait is not heritable were generated as follows for each pathway specific heritability estimate: for severity of neuropathy, residuals and expected values were extracted from linear regression of grade of neuropathy with log cumulative dose of paclitaxel and the first 20 principal components. For each of 1000 permutations, residuals were permuted, summed with expected values for each individual, and used to estimate pathway-specific heritability in GCTA. For onset of neuropathy, deviance residuals were calculated as described, then input as an independent variable in a linear regression including 20 principal components from which residuals and expected values were extracted. As with severity of neuropathy, for each of 1000 permutations, residuals were permuted, summed with expected values for