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Expression of mitochondrial dysfunction-related genes and pathways in paclitaxel-induced peripheral neuropathy in breast cancer survivors

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Abstract

Background: Paclitaxel is one of the most commonly used drugs to treat breast cancer. Its major dose-limiting toxicity is paclitaxel-induced peripheral neuropathy (PIPN). PIPN persists into survivorship and has a negative impact on patient's mood, functional status, and quality of life. No interventions are available to treat PIPN. A critical barrier to the development of efficacious interventions is the lack of understanding of the mechanisms that underlie PIPN. Mitochondrial dysfunction has been evaluated in preclinical studies as a hypothesized mechanism for PIPN, but clinical data to support this hypothesis are limited. The purpose of this pilot study was to evaluate for differential gene expression and perturbed pathways between breast cancer survivors with and without PIPN.

Methods: Gene expression in peripheral blood was assayed using RNA-seq. Differentially expressed genes (DEG) and pathways associated with mitochondrial dysfunction were identified between survivors who received paclitaxel and did (n = 25) and did not (n = 25) develop PIPN.

Results: Breast cancer survivors with PIPN were significantly older; more likely to be unemployed; reported lower alcohol use; had a higher body mass index and poorer functional status; and had a higher number of lower extremity sites with loss of light touch, cold, and pain sensations and higher vibration thresholds. No between-group differences were found in the cumulative dose of paclitaxel received or in the percentage of patients who had a dose reduction or delay due to PIPN. Five DEGs and nine perturbed pathways were associated with mitochondrial dysfunction related to oxidative stress, iron homeostasis, mitochondrial fission, apoptosis, and autophagy.

Conclusions: This study is the first to provide molecular evidence that a number of mitochondrial dysfunction mechanisms identified in preclinical models of various types of neuropathic pain including chemotherapy-induced peripheral neuropathy are found in breast cancer survivors with persistent PIPN and suggest genes for validation and as potential therapeutic targets.

Keywords

Taxanes, mitochondria, neuropathy, breast cancer, survivor, paclitaxel, differential gene expression, pathway analysis

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Introduction

Paclitaxel is one of the most commonly used drugs to treat breast, ovarian, and lung cancers.[1] Its major dose-limiting toxicity is paclitaxel-induced peripheral neuropathy (PIPN). Prevalence rates for PIPN are extremely

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high, ranging from 59% to 87%.[2,3] PIPN is characterized by paresthesias and dysesthesias that occur in a "stocking-glove" distribution. PIPN persists into the cancer survivorship period and has a negative impact on individuals' mood, functional status, and quality of life (QOL).[4] In particular, the negative impact of PIPN on breast cancer (BC) survivors has been identified as a gap in QOL for BC patients.[5,6]

Paclitaxel exerts its primary antitumor effects by binding to beta-tubulin in microtubules, forming extremely stable and nonfunctional microtubules that results in apoptosis.[7] In terms of its neurotoxic effects, the exact mechanisms that underlie the development of PIPN remain unclear. However, several lines of evidence suggest that interruption of microtubule function in neuron impairs axonal transport and results in a dying back neuropathy. In addition, paclitaxel alters mitochondrial function through the depletion of mRNAs that encode the mitochondrial fission/fusion machinery in distal axons.[8] This toxic effect leads to a deficit in axonal energy supply and subsequent axonal degeneration.[9,10]

Using the National Cancer Institute's Common Terminology Criteria for Adverse Events, PIPN of grades 2 to 4 occurs at cumulative doses of between 715 and 1500 mg/m^2.[11] However, not all patients who receive this dose of paclitaxel develop PIPN. The wide range in occurrence rates suggests that genetic factors may play a role in the development of PIPN. While preclinical studies suggest that a number of mechanisms are involved in the development of PIPN,[9,10,12] the majority of the genetic association studies of PIPN evaluated for polymorphisms in candidate genes that influence the metabolism and transport of neurotoxic drugs. As noted in a recent systematic review and meta-analysis of the molecular genetics of chemotherapy-induced peripheral neuropathy (CIPN),[13] notable methodological issues, including lack of standardization and detail in the phenotype definition and acknowledgment of potential confounding factors, prevented the authors from identifying any candidate genes that were associated with CIPN in general or PIPN specifically.

No studies were identified that evaluated for associations between changes in the expression of genes involved in mitochondrial function and chronic PIPN in cancer survivors. Given the preclinical evidence that paclitaxel alters mitochondrial function in primary afferent neurons,[9,10] the purpose of this study was to identify differentially expressed mitochondrial dysfunction (MD)-related genes and perturbed pathways in BC survivors with (n = 25) and without (n = 25) chronic PIPN.

Materials and methods

Survivors and settings

The methods for this study, which is part of a larger study, are described in detail elsewhere.[14] In brief, survivors were recruited from throughout the San Francisco Bay area and met prespecified inclusion and exclusion criteria (see Supplemental Material). The National Coalition for Cancer Survivorship's definition of cancer survivor was used in this study (i.e., a person is a cancer survivor from the moment of diagnosis through the balance of life).[15] Of the 1450 survivors who were screened, 754 were enrolled and 623 completed the self-report questionnaires and the study visit. Data from a randomly selected sample of BC survivors with (n = 25) and without (n = 25) chronic PIPN were used in this analysis.

Study procedures

Research nurses screened and consented the survivors over the phone, sent and asked them to complete the self-report questionnaires prior to their study visit, and scheduled the in-person assessment. At this assessment, written informed consent was obtained, questionnaires were reviewed for completeness, and objective measurements were done. Blood samples were drawn, processed, and stored for subsequent molecular analyses in PAXgene® Blood RNA tubes (Qiagen, Inc.). This study was approved by the institutional review board of the University of California, San Francisco.

Study measures

Demographic and clinical characteristics. Survivors provided information on demographic characteristics and completed the Alcohol Use Identification Test (AUDIT),[16] Karnofsky Performance Status (KPS) Scale,[17–19] and the Self-Administered Comorbidity Questionnaire.[20,21]

Pain measures. Survivors with PIPN rated the intensity of their pain using a 0 to 10 numeric rating scale and completed the pain interference scale from the Brief Pain Inventory.[22] The qualities of PIPN were evaluated using the Pain Quality Assessment Scale.[23,24]

Objective measures of sensation. Light touch was evaluated using Semmes Weinstein monofilaments.[25] Cold sensation was evaluated using the Tiptherm Rod.[26,27] Pain sensation was evaluated using the Neurotip.[27] Vibration threshold was assessed using a vibrometer.[28] For all of the measures of sensation, both the upper and lower extremities on the dominant side were tested.

Balance. Self-report questions from the CIPN Assessment Tool were used to assess the balance.[29] The objective measures of balance were the timed get up and go test (TUG)[30] and the Fullerton Advanced Balance (FAB) test.[31,32]

Phenotypic data analysis

Data were analyzed using SPSS version 23.[33] Descriptive statistics and frequency distributions were calculated for survivors' demographic and clinical characteristics. For the four objective measures of sensation (i.e., light touch, cold, pain, and vibration), composite scores, over all of the sites that were tested on the dominant upper and lower extremities, were created. For light touch, cold, and pain, the number of sites with loss of each sensation was summed. For vibration, the mean score across the sites was calculated. Differences between the PIPN groups in phenotypic characteristics and balance were evaluated using independent sample t tests, χ^2 analyses, Fisher's Exact test (FET), and Mann–Whitney U tests. A p value of $<.05$ was considered statistically significant.

RNA sample preparation

Total RNA was isolated using the PAXgene® Blood miRNA Kit (Qiagen, Inc.) using established procedures.[34] Total RNA from the 50 survivors was sent to the UC Davis Genomics Core Facility for library preparation and for sequencing. Prior to library preparation, 600 ng of total RNA was treated with the Illumina Globin-Zero Gold rRNA Removal Kit (Illumina Inc., San Diego, CA) to deplete cytoplasmic ribosomal RNA[35] and human globin mRNA.[36,37] The globin/ribo depleted RNA was cleaned with Agencourt RNAClean XP (Beckman Coulter, Indianapolis, IN), and the sequencing libraries were prepared with KAPA RNA HyperPrep Kit (Roche Diagnostics Corp., Indianapolis, IN) according to the manufacturer's protocol. Fourteen cycles of polymerase chain reaction (PCR) amplification were used for double six base pair index addition and library fragment enrichment. Prepared libraries were quantified on a Roche LightCycler 480II (Roche Diagnostics Corp.) using KAPA Illumina library quantitative PCR reagents (Roche Diagnostics Corp.).

RNA sequencing (RNA-seq)

Sequencing of the 50 samples was done on an Illumina HiSeq 4000 apparatus (Illumina Inc., San Diego, CA). All 50 samples were multiplexed into a single pool, with each sample labeled with a dual-indexed adapter.[38] The sample pool was sequenced on four lanes for 100 cycles of single-end reads with a 1% PhiX v3 control library spike (Illumina Inc., San Diego, CA). Postsequencing basecall files (bclfiles) were demultiplexed and converted into an FASTQ file format using the bcl2fastq v2.17 software (Illumina Inc., San Diego, CA). Data were posted and retrieved from a secured site hosted by the Core Facility.

RNA-seq alignment, data processing, and quality control (QC)

RNA-seq data processing was performed based on best practices[39,40] and our previous experience.[34,41] Illuimina adapters and leading or trailing low-quality bases were removed, and reads with an average quality per base below 15 in a 4-base sliding window or below a minimum length of 36 bases were removed using trimmomatic.[42] Individual samples were inspected with FASTQC[43] and in aggregate with MultiQC.[44] After initial QC, 10 bases were trimmed from the beginning of all reads and reads were reinspected with FASTQC. The reference genome was prepared using the GRCh38 assembly (gencode.v24.GRCh38.p5.fa).[45] Transcriptome annotations were obtained from the Gencode v24 primary assembly (gencode.v24.primary_assembly.annotation.gtf).[45] Trimmed reads were aligned to the annotated reference genome using the Spliced Transcripts Alignment to a Reference aligner.[46] Output SAM files were validated using ValidateSam. Read groups were added to the SAM file using the Picard tool AddOrReplaceReadGroups. Sorted BAM files were inspected using RNA-SeQC[47] and joined for each sample. Abundance of RNA was estimated from the combined aligned reads using featureCounts.[48]

Replicate count data were processed in edgeR.[49] Ensembl[50] transcripts were annotated with Entrez gene ID and symbol.[51] Lowly expressed tags were filtered out by retaining only those with 3.27 reads per million (10/L where L is the minimum library size in millions) in at least 25 samples (i.e., the smallest group size). Count estimates were normalized with the trimmed means of M values (TMM) method.[52] TMM normalization was applied to the data set in edgeR using calcNormFactors. Data were explored using multidimensional scaling (MDS) plots for all samples to identify sample outliers and potential batch effects due to technical artifacts (i.e., RNA integrity number (RIN), date of RNA extraction). The same technician performed all of the RNA extractions in one laboratory. Associations between technical variables and PIPN group were assessed using Fisher's exact test or a generalized linear model in R. Significance was assessed at a nominal p value of .05. To evaluate the reproducibility of our measurements,[53] we utilized the one additional library[54] that was generated using an alternative preparation protocol (i.e., without globin/ribo depletion step, but from the same RNA materials) and sequenced in the same lane

with the entire sample. We used the weighted kappa statistic to measure the agreement between the count data of this sample produced by the two libraries.[55] The count data were summarized as $\log_2$ counts per million (after TMM normalization) and grouped into 10 levels with a range of 0 to 20 with a bin size of 2.

The technical variables that demonstrated an association with PIPN group were flagged for subsequent evaluation with surrogate variable analysis (SVA). SVA was used to identify variations that contributed to heterogeneity in the sample (e.g., batch effects) which were not due to the variable of interest (i.e., PIPN group membership) or significant demographic covariates.[56] To identify which surrogate variables to include, all surrogate variables were tested for an association with the phenotype and the retained technical variables. Significance was assessed at a nominal p value of .05. Any surrogate variable (SV) that was significantly associated with the phenotype was excluded. Any remaining surrogate variables that were significantly associated with a technical variable were included as covariates in the model for differential expression testing.

Differential expression (DE) analysis

Using edgeR, DE was determined under a variance modeling strategy that addressed the overdispersion observed in gene expression (GE) count data.[57] EdgeR is widely used for DE analysis and performs well relative to other strategies.[58,59] We followed published best practices[60,61] and our previous experience.[34,41] The total sample size is 50 survivors. For this analysis, the overall dispersion and the gene-wise and tag-wise dispersion were estimated using general linear models estimated using the Cox–Reid-adjusted likelihood method.[62,63] Differences between the PIPN groups were tested using likelihood ratio tests. Demographic and clinical characteristics that differed between the PIPN groups, as well as surrogate variables, were included as covariates in the model. We assessed the significance of the transcriptome-wide analysis to identify diffentially expressed genes (DEGs) using a strict false discovery rate (FDR) of 1% under the Benjamini–Hochberg (BH) procedure and no minimal fold-change as evaluated by the topTags and p.adjust R functions.[64,65]

Pathway impact analysis

Pathway analysis approaches have been classified into three classes or generations of approaches (reviewed in [66,67]): overrepresentation analysis (ORA), functional class scoring (FCS), and pathway topology-based (PTB). ORA evaluates for the enrichment of DEGs in a pathway. Although ORA are widely used, but are limited by the predefined set of input genes (i.e., the DE list and the cutoff used to determine it), the statistic used is independent of the measured changes, no differentiation is made between the genes, and all pathways are considered independent. FCS addresses only the first three limitations. In addition, both ORA and FCS consider pathways as lists of genes (i.e., only the counts) and ignore the additional information available in the pathway representation (i.e., the topology). PTB approaches utilize this additional information to evaluate for biological-relevant interactions between genes in the pathway (reviewed in Nguyen et al.[68]). Pathway impact analysis (PIA) is a PTB that implements and impact factor analytic approach, which includes potentially important biological factors (e.g., gene–gene interactions, flow signals in a pathway, pathway topologies) as well as the magnitude (i.e., log fold-change) and the significance (i.e., p values) of the biological differences observed in the DE analysis.[69] We used Pathway Express[70] to perform the PIA. This PTB impact factor analysis approach is widely used and has 1200 citations to date.[68] The analysis included all genes (i.e., cutoff free) and the DE analysis results (i.e., p value and log fold-change) to determine the probability of a pathway perturbation (pPERT). By including all genes in the analysis, and using the DE analysis results to represent the biological differences between the groups, we are also able to capture the adjustments made for the demographic, clinical, and technical (i.e., SVs) variation in the sample. A total of 208 signaling pathways were defined using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.[71] Sequence loci data were annotated with Entrez gene IDs. The gene names were annotated using the HUGO Gene Nomenclature Committee resource database.[72] We assessed for the significance of the pathway analyses using a strict FDR of 1% under the BH procedure.[64,65]

Results

Differences in demographic and clinical characteristics

As shown in Table 1, survivors with PIPN were significantly older ($p = .006$) and were more likely to be unemployed ($p = .022$). In terms of clinical characteristics (see Table 2), survivors with PIPN had a lower AUDIT score ($p = .012$), a higher body mass index (BMI; $p = .017$), and a lower KPS score ($p < .001$). Of note, no between-group differences were found in the total cumulative dose of paclitaxel received or in the percentage of patients who had a dose reduction or delay due to PIPN.

Pain characteristics

Table 3 summarizes the self-reported pain characteristics of the survivors with PIPN. Worst pain severity was

Table 1. Differences in demographic characteristics between breast cancer survivors with and without paclitaxel-induced neuropathy.

	No neuropathy 50.0% (n = 25)	Neuropathy 50.0% (n = 25)	
Characteristic	Mean (SD)	Mean (SD)	Test, p value
Age (years)	52.2 (9.5)	60.0 (9.4)	t = −2.89, p = .006
Education (years)	16.6 (2.4)	16.3 (2.9)	t = 0.32, p = .750
	% (n)	% (n)	
Married/partnered	80.0 (20)	76.0 (19)	FE, p = 1.000
Lives alone	16.0 (4)	12.0 (3)	FE, p = 1.000
Employed	72.0 (18)	36.0 (9)	FE, p = .022
Ethnicity			
White	80.0 (20)	76.0 (19)	FE, p = 1.000
Nonwhite	20.0 (5)	24.0 (6)	
Annual household income			
<$30,000	16.7 (4)	19.0 (4)	
$30,000–$69,999	16.7 (4)	19.0 (4)	U, p = .317
$70,000–$99,999	16.7 (4)	33.3 (7)	
>$100,000	50.0 (12)	28.6 (6)	
Child care responsibilities	25.0 (6)	20.0 (5)	FE, p = .742
Adult care responsibilities	4.3 (1)	4.3 (1)	FE, p = 1.000

FE: Fisher's exact test; SD: standard deviation; U: Mann–Whitney U test.

reported as 6.3 (±2.1) of the 10, and the duration of PIPN was 3.8 (±3.9) years.

Differences in sensation

Survivors with PIPN had a higher number of lower extremity sites with loss of light touch, cold, and pain sensations (all, $p < .05$). Vibration thresholds were significantly higher in the PIPN group ($p = .009$, Table 4).

Differences in balance

Survivors with PIPN were more likely to report trouble with balance ($p < .001$) as well as higher severity and distress (both $p < .05$) scores associated with balance problems (Table 4). In addition, these survivors reported worse TUG ($p = .001$) and worse FAB ($p = .004$) scores.

GE measurements

Mean RIN was 8.27 (minimum = 7.2), median sequenced library size was 27,458,032, and median aligned library size was 4,412,000 reads. The within-replicate agreement (kappa = 0.82) was consistent with high agreement measures observed in a recent study (i.e., 0.62–0.81) that evaluated the variation of technical replicates within RNA-seq data using non-clinical (i.e., *Drosophila*) samples.[55] After QC filtering, 12,491 transcripts were retained from the total of 60,725 GENCODE transcript targets. No samples were identified as outliers in the MDS plots. No batch effects were identified for sample preparation order, RIN value, or RNA processing date in the first four dimensions of MDS plots. PIPN group was associated with the RNA extraction date (FET p value = .01) but not with RIN score or level. RNA extraction date was strongly associated with one of the six identified surrogate variables. This surrogate variable was included in the model as a covariate to control for potential batch effects. The phenotype characteristics that were associated with PIPN group membership (i.e., KPS, employment, age, BMI, and AUDIT score) were included as covariates in the model. The overall dispersion was observed to be 0.076 (biological coefficient of variation = 0.276), which is lower than values reported in other clinical data sets.[62] Using RNA-seq power,[73] we estimate that with 25 replicates in each condition (i.e., with and without PIPN), an average depth of coverage of 15 million reads per replicate, and the observed biological coefficient of variation (i.e., 0.276), we are powered to detect 1.5-fold changes for 83% of genes, and 2-fold changes for 99% of genes, at a type I error rate of 0.01.

Differences in whole-transcriptome GE

The DE analysis included 11,487 genes. After correction for multiple hypothesis testing at a conservative FDR of 1% (adjusted p value < .01), 20 genes were identified as differentially expressed between the survivors with and without PIPN. Of these, four genes were related to MD (Table 5).

Successful annotation with ENTREZ IDs was performed for 11,174 unique genes. Fold changes and

Table 2. Differences in clinical characteristics between breast cancer survivors with and without paclitaxel-induced peripheral neuropathy.

Characteristic	No neuropathy 50.0% (n=25) Mean (SD)	Neuropathy 50.0% (n=25) Mean (SD)	Test, p value
Karnofsky Performance Status score	92.9 (6.2)	80.0 (11.2)	t = 5.02, p <.001
Body mass index (kg/m^2)	23.5 (3.5)	27.2 (6.5)	t = −2.50, p = .017
Number of comorbidities	1.3 (1.2)	1.5 (1.7)	t = −0.55, p = .589
Self-Administered Comorbidity Questionnaire score	2.7 (2.7)	3.6 (4.2)	t = −0.88, p = .384
AUDIT score	3.0 (1.7)	1.7 (1.8)	t = 2.63, p = .012
Years since cancer diagnosis	4.2 (4.7)	4.6 (3.9)	t = −0.34, p = .739
Number of prior cancer treatments	3.6 (0.8)	3.6 (0.8)	t = −0.17, p = .863
Number of current cancer treatments	0.7 (0.7)	0.6 (0.7)	t = 0.61, p = .543
Number of metastatic sites (out of seven)	1.0 (0.8)	0.6 (0.5)	t = 1.72, p = .092
Number of metastatic sites without lymph node involvement	0.2 (0.6)	0.04 (0.2)	t = 0.92, p = .365
	% (n)	% (n)	
Smoker (ever)	37.5 (9)	32.0 (8)	FE, p = .769
Exercise on a regular basis (% yes)	87.5 (21)	60.0 (15)	FE, p = .051
Born prematurely (% yes)	0.0 (0)	12.5 (3)	FE, p = .110
Comorbid conditions (% yes)			
Osteoarthritis	8.0 (2)	28.0 (7)	FE, p = .138
Back pain	28.0 (7)	24.0 (6)	FE, p = 1.000
Depression	28.0 (7)	16.0 (4)	FE, p = .496
High blood pressure	12.0 (3)	28.0 (7)	FE, p = .289
Heart disease	0.0 (0)	4.0 (1)	FE, p = 1.000
Diabetes	0.0 (0)	8.0 (2)	FE, p = .490
Lung disease	4.0 (1)	4.0 (1)	FE, p = 1.000
Anemia or blood disease	0.0 (0)	0.0 (0)	Test not run
Ulcer or stomach disease	0.0 (0)	4.0 (1)	FE, p = 1.000
Kidney disease	0.0 (0)	0.0 (0)	Test not run
Liver disease	0.0 (0)	4.0 (1)	FE, p = 1.000
Rheumatoid arthritis	0.0 (0)	4.0 (1)	FE, p = 1.000
Total cumulative dose of taxol (mg/m^2)**	782.8 (228.6)	814.7 (217.0)	t = 0.14, p = .893
Patients who had a dose reduction or delay due to neuropathy (% (n))**	0.0 (0)	12.0 (3)	FE, p = .235

Note: AUDIT: Alcohol Use Disorders Identification Test; FE: Fisher's exact test; kg: kilograms; m^2: meters squared; mg: milligrams; SD: standard deviation.
**Doses are reported as milligrams per meter squared.

p values from the DE analysis for these genes were included in the pPERT analysis of the 208 KEGG signaling pathways. PIA identified 53 KEGG signaling pathways that were significantly perturbed between the PIPN groups after correction for multiple hypothesis testing at a conservative FDR of 1% (adjusted perturbation p value <.01). Of these, 10 KEGG signaling pathways were related to MD (Table 6; Figure 1).

Discussion

This study is the first to provide molecular evidence that some of the mitochondrial mechanisms identified in preclinical models of PIPN[12,74,75] are found in cancer survivors. While a number of mechanisms are hypothesized to underlie the development of PIPN,[10] the mitotoxicity hypothesis proposes that damage to primary afferent sensory neurons leads to impairments in a variety of mitochondrial functions.[76] None of the previously published candidate gene or genome-wide association studies identified any MD-related genes. In this study, we found support for the differential expression of genes and significant perturbations in a number of pathways associated with MD including oxidative stress, iron homeostasis, mitochondrial fission, and apoptosis and autophagy.[10]

Oxidative stress

Mitochondria play a vital regulatory role in cellular physiology. Within neurons, more than 90% of the mitochondria are localized in axons where they are necessary for energy generation.[76,77] Paclitaxel opens the mitochondrial permeability transition pore, which is associated with increased generation of reactive oxygen

Table 3. Pain characteristics of the breast cancer survivors with paclitaxel-induced peripheral neuropathy.

	Lower extremity (n = 25)
Characteristic	Mean (SD)
Pain characteristics	
Duration of neuropathy (years)	3.8 (3.9)
Pain now	3.1 (2.2)
Average pain	3.6 (2.0)
Worst pain	6.3 (2.1)
Days per week in pain	4.0 (3.2)
Hours per day in pain	13.0 (8.8)
Pain Interference Scale (0–10)	
Walking ability	4.2 (3.2)
Balance	4.0 (3.1)
General activity	3.0 (2.9)
Enjoyment of life	2.9 (2.8)
Sleep	2.9 (3.0)
Normal work	2.8 (3.0)
Mood	1.9 (2.0)
Relations with other people	1.5 (1.9)
Sexual activity	0.8 (1.9)
Mean interference score	2.7 (2.2)
Pain Qualities Assessment Scale scores (0–10)	
Numb	5.8 (3.0)
Tingling	4.8 (3.1)
Unpleasant	4.6 (2.3)
Dull	3.6 (3.3)
Intense	3.5 (2.5)
Electrical	2.9 (3.2)
Cramping	2.8 (3.3)
Aching	2.5 (2.6)
Hot	2.4 (3.1)
Radiating	2.3 (3.0)
Shooting	2.2 (3.1)
Sharp	2.0 (2.5)
Heavy	2.0 (2.7)
Tender	1.7 (2.3)
Throbbing	1.6 (2.6)
Sensitive skin	1.4 (1.8)
Itchy	1.0 (1.6)
Cold	0.9 (1.7)
Intense—surface pain	3.7 (3.0)
Intense—deep pain	3.5 (2.9)

SD: standard deviation.

species (ROS).[76] This type of oxidative stress leads to structural and functional damage to mitochondria. In animal studies, the administration of paclitaxel was associated with the appearance of swollen and vacuolated mitochondria in peripheral nerves and dorsal root ganglion (DRG) cells.[12,78]

In this study, we found differentially expressed genes (i.e., peroxiredoxin 5 (*PRDX5*), glutaredoxin 5 (*GLRX5*)) and perturbed pathways (i.e., HIF-1 signaling pathway,[79] PI3K-Akt signaling pathway,[80] Peroxisome[81]) that are regulated or mediated by oxidative stress. Several lines of preclinical evidence support our clinical findings.

While not specifically evaluated in PIPN, in murine models of peripheral nerve injury, the generation of ROS is upregulated, and antioxidant pathways are downregulated or functionally impaired.[82,83] This redox imbalance is enhanced by mitochondrial damage.[84] However, most neurons survive this imbalance, which suggests that endogenous defense systems are activated to restore homeostasis. Peroxiredoxin, glutaredoxins, and thioredoxins are part of this endogenous defense system that remove ROS and mediate the response to the redox stress.[85] In one study that evaluated changes in mRNA in DRG cell bodies and axons after unilateral sciatic nerve injury (SNI),[86] enriched mRNAs in the axonal compartment on the side of the SNI included mitochondrial genes as well as genes of the peroxidase family including Prdx5 (the rat homolog of human *PDRX5*, which is downregulated in our survivors with PIPN in this study).

In another study that evaluated the effects of thioredoxin-fold proteins in an SNI model,[87] while both glutaredoxin (i.e., Glrx5) and peroxiredotoxins (i.e., Prdx4, Prdx5) were found in the DRG, only Prdx4 and Prdx5 were upregulated following the SNI. Of note, Prdx5 upregulation was associated with an increase in respective mRNA and protein accumulation in peripheral fibers proximal to the injury. This finding is consistent with an accumulation of mitochondria as a result of blocked axonal transport.[87] In addition, this upregulation of Prdx5 was dependent on the presence of endogenous HIF-1 (a perturbed pathway in our survivors with PIPN), a global regulator of oxygen homeostasis that facilitates oxygen delivery and adaptation to oxygen deprivation.[88,89] The authors hypothesized that failure of Prdx5 upregulation increases the risk for chronic neuropathy.[87] Interestingly, differences in *PDRX5* GE were observed in sural nerves from patients with progressing compared to nonprogressing diabetic neuropathy.[90]

Iron homeostasis

Perturbation in the ferroptosis pathway and the decreased expression of *GLRX5* was found in our cancer survivors with PIPN. Ferroptosis is a regulated cell death driven by lipid ROS[91] and is characterized by abnormal mitochondria morphology.[92] Iron is an important cofactor in metabolically active cells like neurons. A carefully controlled supply of iron is needed for mitochondrial function, axonal transport, and myelination.[93–95] While glutaredoxins (e.g., *GLRX5*) are involved in redox reactions,[85] they are needed for iron homeostatsis.[96,97] A growing body of evidence suggests that alterations in mitochondrial iron metabolism are

Table 4. Differences in sensation and balance measures between breast cancer survivors with and without paclitaxel-induced peripheral neuropathy.

Characteristic	No neuropathy 50.0% (n = 25) Mean (SD)	Neuropathy 50.0% (n = 25) Mean (SD)	Statistic; p value
Sensation measures[a]			
Light touch—lower extremity sites (out of nine)[b]	0.1 (0.3)	1.5 (1.5)	t = −4.42, p <.001
Cold—lower extremity sites out of four[c]	1.5 (1.3)	2.3 (1.0)	t = −2.54, p = .014
Pain—lower extremity sites (out of nine)[d]	1.6 (1.6)	3.0 (2.1)	t = −2.71, p = .009
Vibration—lower extremity sites (volts)[e]	16.2 (8.1)	25.1 (14.0)	t = −2.76, p = .009
Balance measures			
Trouble with balance (% yes (n))[f]	13.0 (3)	76.0 (19)	FE, p <.001
Severity of balance trouble (0–10)[g]	1.7 (1.2)	5.6 (2.9)	t = −4.16, p = .004
Frequency of balance trouble (0–10)[h]	2.3 (1.5)	5.1 (3.2)	t = −2.37, p = .060
Distress from balance trouble (0–10)[i]	1.7 (1.2)	5.8 (3.2)	t = −4.17, p = .003
Timed get up and go test (>13.5 s = higher risk for falls)	6.1 (1.1)	8.6 (3.1)	t = −3.76, p = .001
Fullerton Advanced Balance test (≤25 is associated with a higher risk of falls)	38.0 (3.1)	33.4 (6.7)	t = 3.14, p = .004

Note: FE: Fisher's exact test; SD: standard deviation.

[a]Changes in sensation are reported for the dominant extremity.

[b]Lower extremity sites for light touch were as follows: pad of great toe, pad of third toe, pad of fifth toe, base of heel, metocarpophalangeal (MP) joint of great toe, MP joint of third toe, MP joint of fifth toe, midway along tibia, and patella.

[c]Lower extremity sites for cold were as follows: top of great toe at first MP joint, pad of great toe, dorsum of foot midpoint, and medial malleolus.

[d]Lower extremity sites for pain were as follows: pad of great toe, pad of third toe, pad of fifth toe, base of heel, MP joint of great toe, MP joint of third toe, MP joint of fifth toe, midway along tibia, and patella.

[e]Lower extremity sites for vibration were as follows: dorsal IP joint of great toe, medial malleolus, and patella.

[f]Since your chemotherapy, have you had trouble with your balance?

[g]At its worst, how severe is the trouble with your balance (0 = not at all severe to 10 = extremely severe)?.

[h]How often do you have trouble with your balance (0 = never to 10 = always)?

[i]At its worst, how distressing is the trouble with your balance (0 = not at all distressing to 10 = extremely distressing)?

Table 5. Differentially expressed mitochondrial dysfunction-related genes between breast cancer survivors with and without paclitaxel-induced peripheral neuropathy (PIPN).

Ensemble gene ID	Gene symbol	Name	Entrez ID	logFC[a]	Adjusted p value[b]
ENSG00000126432	*PRDX5*	Peroxiredoxin 5	25824	−0.987	.0098
ENSG00000182512	*GLRX5*	Glutaredoxin 5	51218	−1.703	.0062
ENSG00000214253	*FIS1*	Fission, mitochondrial 1	51024	−1.062	.0062
ENSG00000170315	*UBB*	Ubiquitin B	7314	−1.374	.0062

[a]logFC: Fold change of the $\log_2$ transformed normalized gene expression counts between the groups. A negative logFC denotes lower expression in survivors with PIPN as compared to those without PIPN.

[b]p Value adjusted using the Benjamini–Hochberg method.

Table 6. Significantly perturbed mitochondrial dysfunction-related Kyoto Encyclopedia of Genes and Genomes pathways between breast cancer survivors with and without paclitaxel-induced peripheral neuropathy.

Pathway ID	Pathway name	Total perturbation	Adjusted pPert[a]
hsa04137	Mitophagy–animal	11.79	.004
hsa04150	mTOR signaling pathway	9.60	.004
hsa04152	AMPK signaling pathway	8.99	.004
hsa04151	PI3K-AKT signaling pathway	8.04	.004
hsa04066	HIF-1 signaling pathway	7.45	.004
hsa04216	Ferroptosis	6.98	.004
hsa04115	p53 signaling pathway	5.68	.007
hsa04146	Peroxisome	5.32	.007
hsa04068	FoxO signaling pathway	5.32	.008
hsa04218	Cellular senescence	5.36	.008

[a]pPert: Perturbation p value adjusted using the Benjamini–Hochberg method.

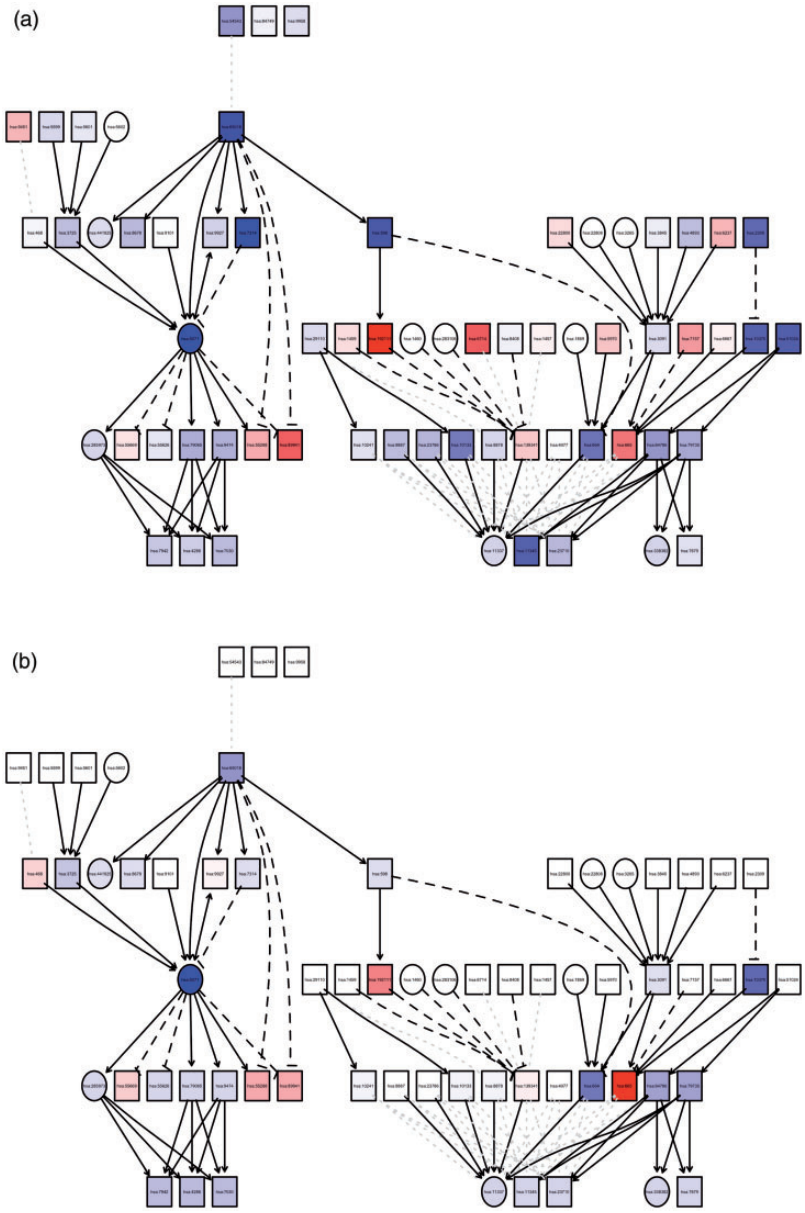

Figure 1. Graph summary of pathway level statistics. (a) The measured expression change versus (b) perturbation accumulation in the Mitophagy–Animal Kyoto Encyclopedia of Genes and Genomes pathway (hsa04137). The square nodes denote genes with gene expression change, and the circle nodes denote all other nodes. The color of each node represents the perturbation (red = positive, blue = negative), and the shade represents the strength of the perturbation. Note that the square nodes with no parents have no accumulation.

involved in a number of neurological disorders (e.g., Parkinson's disease) as well as in motor and cognitive decline associated with aging.[98] In addition, the products of iron-regulatory and iron-transport-pathway genes may play a role in the development of diabetic[99,100] and HIV neuropathy.[101]

While no preclinical studies have identified an association between CIPN and ferroptosis, an interesting finding that warrants additional investigation is related to the mitochondrial iron-binding protein called frataxin (*FXN*). This protein is critical for mitochondrial iron metabolism, overall cellular iron homeostasis, and antioxidant protection.[102–104] In an in vitro study of CIPN,[105] the administration of alpha-lipoic acid increased the expression of frataxin in vehicle-treated DRG cells as well as in DRG cells treated with paclitaxel or cisplatin. The authors concluded *FXN* may play a key role in neuroprotective pathways.

Mitochondrial fission

Mitochondrial fission is the process in which mitochondria are divided into smaller units. Mitochondrial fission

enhances the number of mitochondria in neural cells and helps to maintain their energy demands.[77,106] Fission mitochondrial 1 (*FIS1*, which was downregulated in survivors with PIPN) is a primary mediator of mitochondrial fission and has been implicated in MD and autophagy.[107,108] *FIS1* recruits dynamin-related protein 1 (*DLRP1*/*Drp1*) from the cytosol, [109] which is the primary executor of fission.[106] Defects in mitochondrial fission are implicated in a number of neurodegenerative disorders (e.g., hereditary optic neuropathy, Parkinson's disease).[106,110,111]

Evidence from preclinical studies supports the association found in our cancer survivors. In a study that evaluated whether Drp1 catalyzed the process of mitochondrial fission, Sprague Dawley rats were treated with 2′,3′-dideoxycitidine (ddC) or oxaliplatin.[112] In this study, intrathecal administration of an oligodeoxynucleotide antisense against Drp1 resulted in a decrease in its expression in peripheral nerves and markedly attenuated neuropathic hyperalgesia associated with both drugs. In a more recent study that evaluated the role of Drp1 in neuropathic pain induced by perineural human immunodeficiency virus gp120,[113] intrathecal administration of an oligodeoxynucleotide antisense against Drp1 decreased mechanical allodynia and decreased the spinal expression of increased Drp1 protein induced by gp120.

While not studied in terms of neuropathic pain, the interconnection between mitochondria and the peroxisome (a perturbed pathway in our cancer survivors) is an area of intense investigation.[81,114,115] Peroxisomes represent a class of ubiquitous and dynamic single membrane bound organelles in eukaryotic cells.[114] Substantial evidence suggests that peroxisomes and mitochondria have a close functional interplay. In particular, peroxisomes and mitochondria contribute to ROS homeostasis and share a redox-sensitive relationship.[114] It is plausible that this pathway could be involved in PIPN.

Apoptosis and autophagy

Several signaling pathways involved in mitochondrial apoptosis and autophagy (i.e., PI3K-Akt, mTOR, FoxO, and AMPK) were perturbed in this study. In terms of the PI3K/Akt/mTOR pathways, several lines of evidence suggest that they are involved in the development of neuropathic pain.[116] For example, in a rat model of chronic constriction injury (CCI),[117] compared to control and sham animals, increased mRNA expression and protein levels were observed for PI3K, Akt, and mTOR in the CCI animals. In a more recent study that evaluated for changes in PI3K/Akt signaling in spinal cord slice preparations following CCI,[118] PI3K/Akt signaling increased in the L4-L6 spinal cord sections. The authors concluded that PI3K/Akt signaling is required for central sensitization in the CCI neuropathic pain model.

The FoxO signaling pathway, that is regulated by the PI3K, Akt, and mTOR signaling pathways,[119] is a potential target for a number of neurodegenerative disorders [120] including diabetes.[121] In a recent study that evaluated for changes in genes and pathways associated with neuropathic pain using a bioinformatics approach,[122] upregulated genes in the FoxO signaling pathway were associated with spinal nerve ligation.

Recent evidence suggests that AMPK may be a potential target for the treatment of chronic pain.[123–125] AMPK and peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) signaling axes sense the mitochondrial demands of the cell and regulate mitochondrial function.[126] In a recent preclinical study,[127] phosphorylation and expression of AMPK/PGC-1α and mitochondrial chain complex proteins were downregulated in the DRG of streptozotocin-diabetic rats. In addition, the administration of resveratrol (i.e., a polyphenol that augments APMK phosphorylation and axonal growth[128]) was associated with the reversal of thermal hypoalgesia, attenuation of foot skin intradermal fiber loss and a reduction in myelinated fiber mean axonal caliber in streptozotocin-diabetic rats. The authors concluded that the development of neuropathy was linked to nutrient excess and MD through defective signaling of the AMPK/PGC-1α pathway. In terms of CIPN, the coadministration of metformin (i.e., a widely used antidiabetic drug that activates AMPK[129]) with cisplatin or paclitaxel prevented the occurrence of mechanical allodynia.[130] In addition, metformin prevented the cisplatin-induced increase in the latency to detect an adhesive patch (a measure of sensory deficits) and the reduction in the density of intradermal nerve fibers in the paw.

Perturbations in the p53 signaling pathway were identified in our cancer survivors with PIPN. This pathway regulates a complex transcriptional program involved in a variety of biological processes, including cell cycle arrest and apoptosis.[131] Mitochondrial translocation of the p53 molecule is associated with MD in a number of neurological disorders.[132,133] In terms of CIPN, cisplatin treatment rapidly increased mitochondrial levels of p53 in DRG neurons as well as reduced mitochondrial membrane potential and disrupted mitochondrial integrity and function.[134] In addition, the administration of the mitochondrial protectant pifithrin-μ (PFT-μ; which prevents the mitochondrial accumulation of p53) prevented both paclitaxel- and cisplatin-induced mechanical allodynia and sensory loss.[135] Equally important, in other studies, the administration of PFT-μ protected against loss of mitochondrial membrane potential,

abnormalities in mitochondrial morphology, and functional mitochondrial deficiencies in DRG neurons.[12,134,135]

In the current study, perturbations in two-related pathways (i.e., mitophagy and HIF-1 signaling) and decreased expression of a related gene (i.e., ubiquitin) were identified in survivors with PIPN. Ubiquitination of mitochondrial proteins, including HIF-1A transcription factor,[136] regulates many aspects of mitochondrial homeostasis including mitophagy.[137] Mitophagy, a selective form of autophagy, mediates the selective removal of mitochondria[138] and plays an essential role in mitochondrial homeostatis.[139] While no studies were identified that found associations between CIPN and ubiquitination or mitophagy, alterations in these two processes are associated with neurodegeneration.[140]

Limitations

Several limitations warrant consideration. While our sample size was relatively small, we had an extremely well-characterized sample of BC survivors with and without PIPN. Of note, no differences were found in the total cumulative dose of paclitaxel that these survivors received. Given that one cannot test for differences in RNA in DRG neurons from living patients, as have others evaluating for GE differences in patients with painful neuropathies,[141,142] we have evaluated for differences in RNA expression from peripheral blood. Because our molecular analyses were done using blood samples, we can only infer that the perturbations identified are consistent with changes within the peripheral nervous system. Our findings warrant confirmation in an independent sample. However, to decrease the possibility of spurious associations, we sequenced RNA to a considerable depth, applied best practices for the RNA-seq analyses, followed a strict QC procedure, and evaluated for significance with a severely stringent FDR.

Future research

This study is the first to provide molecular evidence that a number of mitochondrial mechanisms identified in preclinical models of various types of neuropathic pain, including CIPN,[12,74,75] are found in cancer survivors with persistent PIPN. The limitations of the analyses of RNA from blood and nonneural tissue notwithstanding, we did observe differential profiles of expression and perturbation in these processes, which suggest persistent damage and/or changes in the regulation of mitochondria. Future studies need to evaluate for differences in epigenetic changes (i.e., methylation, microRNA) between survivors with and without PIPN, which may reflect changes in regulation patterns. In addition, our findings suggest that chronic PIPN involves multiple MD-related mechanisms. Therefore, studies are warranted that evaluate for common and distinct MD-related mechanisms associated with other neurotoxic chemotherapy drugs (e.g., platinum, platinum, and taxane combination). It must be acknowledged that a cross-sectional study cannot demonstrate causality. Longitudinal studies are needed that evaluate for changes over time in the molecular mechanisms associated with CIPN. These longitudinal studies need to include an in-depth characterization of the CIPN phenotype as well as pretreatment and multiple posttreatment assessments of a number of molecular markers (i.e., genes, GE, epigenetics). Translation of these findings, if validated, may be accelerated by investigating drugs that target mitochondrial function to be repurposed to treat CIPN.[143] The use of sophisticated bioinformatics approaches and network analyses may allow us to determine the timing of the disruptions in mitochondrial function associated with neurotoxic chemotherapy so that interventions can be initiated to prevent this dose-limiting and devastating adverse effect.

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Supplemental Material

Supplemental material is available for this article online.

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