Longitudinal RNA sequencing of skin and DRG neurons in mice with paclitaxel-induced peripheral neuropathy

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Data Note

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Abstract

Paclitaxel-induced peripheral neuropathy is a condition of nerve degeneration induced by chemotherapy, which afflicts up to 70% of treated patients. Therapeutic interventions are unavailable due to an incomplete understanding of the underlying mechanisms. We previously discovered that major physiological changes in the skin underlie paclitaxel-induced peripheral neuropathy in zebrafish and rodents. The precise molecular mechanisms are only incompletely understood. For instance, paclitaxel induces the upregulation of MMP-13, which when inhibited prevents axon degeneration. To better understand other gene regulatory changes induced by paclitaxel, we induced peripheral neuropathy in mice following intraperitoneal injection either with vehicle or paclitaxel every other day four times total. Skin and dorsal root ganglion neurons were collected based on distinct behavioural responses categorized as “pain onset” (d4), “maximal pain” (d7), “beginning of pain resolution” (d11) and “recovery phase” (d23) for comparative longitudinal RNA sequencing. The generated datasets validate previous discoveries and reveal additional gene expression changes that warrant further validation with the goal to aid in development of drugs that prevent or reverse paclitaxel-induced peripheral neuropathy.

Background & Summary

Peripheral neuropathy is a common side effect of chemotherapy characterized by paraesthesia (tingling), numbness, pain, temperature sensitivity, and motor weakness. Paclitaxel (Taxol) is one of the most widely used chemotherapeutic agents, which primarily affects the somatosensory neurons innervating the skin[1–3]. Pathological examinations suggest that intraepidermal unmyelinated axons are the first to degenerate upon paclitaxel treatment[4–8]. Thus, understanding the genetic mechanisms underlying the earliest manifestations of the disease will be essential to develop therapies that allow chemotherapy patients to complete cancer treatment without disruption, and prevent irreversible long-term symptoms.

Few studies have established expression profiles of chemotherapy-induced peripheral neuropathy. In one study, parallel gene expression profiles from dorsal root ganglion (DRG) neurons in mice were established following injection with the chemotherapeutic agents, oxaliplatin, vincristine, and cisplatin[9]. This comparative study revealed that only few genes were common among these data sets, suggesting that fundamental differences in the aetiology of chemotherapy-induced peripheral neuropathy (CIPN) must exist. This may not be surprising given the differences in the mechanisms of action for each of these chemotherapeutic agents, leading to potentially different off-target effects. In addition, the investigation of dorsal root ganglion (DRG) neurons may have obscured common upstream mechanisms. For instance, we previously showed that sensory neurons are secondarily affected by earlier epidermal damage, which promotes the degeneration of intraepidermal nerve endings in zebrafish, rats, and mice treated with paclitaxel. Epidermal keratinocytes are damaged due to increased reactive oxygen species formation and upregulation of matrix metalloproteinases, such as MMP-13, leading to extracellular matrix damage that ultimately affects the axons, leading to their degeneration[8]. Epidermal damage can be prevented when animals are treated with pharmacological MMP-13 inhibitors[10]. Therefore, the skin plays a crucial role in sensory axon homeostasis.

Existing genomic studies have focused on single time point analyses and single cell types[11,12]. For instance, RNA sequencing was used to analyse blood samples of breast cancer survivors who suffered from long-term paclitaxel-induced peripheral neuropathy and these samples were compared to breast cancer survivors without neuropathy[13]. This study identified changes in mitochondrial genes that had been previously identified in preclinical CIPN models as differentially regulated, validating the importance of these models in studying the human pathology[13]. Potentially, mitochondrial dysfunction might play a role in the deficiency of some patients to resolve their neuropathy symptoms. Despite these findings, longitudinal studies to detect changes in affected tissues over prolonged time periods have not been conducted, and thus no data is available on gene expression changes prior to the onset of neuropathic symptoms. This information, however, will be critical to understand the molecular gene expression networks involved in the onset, progression, and resolution of neuropathy.

To address this need, we performed a comprehensive RNAseq study using skin and DRG neuron samples of vehicle and paclitaxel-treated mice. We compared gene expression profiles according to pain profiles generated in these mice. Mice were injected 4 times every other day with either vehicle or paclitaxel and subsequently underwent a recovery period between day 7 and day 23. Tissues were collected and analysed during these time points, which were categorized as “pain onset” on day 4, “maximal pain sensitivity” on day 7, “beginning of pain resolution” at day 11, and “post pain” on day 23. The generated data sets will be useful for the research community to further validate the genes implicated in paclitaxel-induced peripheral neuropathy.

Methods

Animals

All animal procedures were approved by the University of New England Institutional Animal Care and Use Committee. Adult male C57BL6/J mice (JAX) weighing 20–25 grams were purchased from the Jackson Laboratory. Upon arrival, mice were housed 4/cage and allowed to acclimate to the facility for 7 days. All animals were kept on a 12-hour light/dark cycle with *ad libitum* access to food and water.

Paclitaxel Treatment

The experimental design, time-points and downstream analyses are depicted in Fig. 1a. Paclitaxel was administered on days 0, 2, 4 & 6. Paclitaxel (Sigma-Aldrich) was dissolved in (1:1 Cremophor:Ethanol) and further diluted in 0.9% NaCl to make a final concentration of 0.4mg/ml. Mice were injected intraperitoneally with either vehicle or paclitaxel at a volume of 10ml/kg bodyweight to make a final concentration of 4mg/kg (cumulative 16mg/kg).

Analysis of Tactile Thresholds

Tactile allodynia was quantified (in the mornings prior to injections when assessed on injection days) by measuring the hind paw withdrawal threshold to von Frey filament stimulation, using the up-down method as previously reported[14]. Results are shown in Fig. 1b. Animals were placed in a clear Plexiglas chamber and allowed to habituate for ~60 minutes. Touch-Test filaments (North Coast Medical, CA) were used for all testing. The filament range was: 2.44, 2.83, 3.22, 3.61, 4.08, 4.31, 4.56, starting with 3.61. Withdrawal thresholds were determined by sequentially increasing and decreasing the stimulus intensity ("up and down" method). This up-down method was stopped four measures after the first positive response. The response threshold was subsequently analysed by using a Dixon nonparametric test and expressed as the paw withdrawal threshold in gram force values[15]. A clear paw withdrawal, shaking or licking was considered as a positive or painful response.

Tissue collection

Animals were exsanguinated by intracardial perfusion of 100ml ice cold heparinised Phosphate buffered saline (PBS). Tissues were then immediately harvested on ice before flash freezing and storage at −80°C. DRG neurons were harvested from left lumbar segments L3, L4 and L5. Plantar skin was taken from the left hind paw. Tissues were harvested at timepoints consistent with the development of paclitaxel-induced neuropathy in rodents characterised as “pain onset” on day 4, “maximal pain sensitivity” on day 7, “beginning of pain resolution” at day 11, and “post pain” on day 23[100].

RNA extraction and quality control

DRG were homogenised using a glass-Teflon homogeniser on ice for 2 minutes with 350µl buffer RLT plus 2-Mercaptoethanol (Qiagen). RNA was extracted using Qiagen RNeasy microcolumns according to the manufacturer's protocol and eluted with 30µl ultra-pure RNase-free water. Skin was homogenised with a rotor-stator homogeniser for 2 minutes on ice with TRIzol reagent (Thermo) before phase separation with phase lock gel microcentrifuge tubes (Eppendorf) to prevent guanidine salt contamination, according to the manufacturer's protocol. RNA purity was confirmed using a nanodrop UV spectrometer (260/280 ratio >2), and RNA quality was assessed with an Agilent bioanalyzer (RIN > 7).

cDNA library preparation and sequencing

Ribosomal RNA-depleted cDNA libraries were prepared using KAPA RNA HyperPrep kits with RiboErase (KAPA Biosystems) according to the manufacture’s protocol. Libraries were sequenced with an Illumina Hi-seq 3000 (single-end, 1 X 100 bp).

Sequence alignment, counts and quality control

Remaining adapters and poor quality sequences were trimmed with Trimmomatic (version 0.38)[16] with the following parameters: ILLUMINACLIP:TruSeq3-SE.fa:2:30:10 SLIDINGWINDOW:4:15 MINLEN:15. Sequence quality was then verified using FastQC and summarised with MultiQC (version 1.9) (Fig. 1c). Using STAR[17] (version 2.7.5a_2020-06-19), sequences were aligned to mouse genome assembly m38 with Ensembl version 100 annotations using the –quantMode GeneCounts parameter. Mapping quality was verified using MultiQC (Fig. 1d-e).

Data Records

All raw and normalized gene count matrices produced by DESeq2 were deposited in the NCBI Gene Expression Omnibus (GEO) database under accession number GSE185084. This GEO entry includes links to the raw count data in .txt.bz2 format. Each processed matrix data file of raw or normalized counts contains a header row corresponding to the sample followed by 52,391 rows, each corresponding to a unique transcript. **Table 1** describes each of the individual raw files, their corresponding sample ID, and a description of each sample according to its timepoint, treatment, and tissue type.

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The GEO accession (GSE185084) can be reviewed under: https://nam10.safelinks.protection.outlook.com/?url=https%3A%2F%2Fwww.ncbi.nlm.nih.gov%2Fgeo%2Fquery%2Facc.cgi%3Facc%3DGSE185084&data=04%7C01%7Cacirrincione%40miami.edu%7C1e0
Enter token alutiykevzedtwv into the box

Technical Validation

Differential expression analysis

Gene counts from STAR alignment were imported into the R environment using Rstudio (R Version 4.0.3; R studio version 1.3.1093) and the package *base* version 4.0.3. The package *dplyr* version 1.0.5 was used to construct individual raw gene count matrices per tissue type, skin and dorsal root ganglion neurons (DRGs), each consisting of 32 samples combined from both treatment groups. Normalized gene expression for both tissue data sets was calculated using the default parameters in the Deseq2 package version 1.3.1.

Expression of housekeeping and validation genes

Normalized gene counts were plotted using *ggboxplot* from the *ggpubr* package version 0.4.0 using Rstudio (R Version 4.0.3; R studio version 1.3.1093). Previously validated housekeeping genes for DRG and skin were plotted in addition to genes shown to be overexpressed during paclitaxel-induced peripheral neuropathy in either one. To validate DRG-specific housekeeping genes, we plotted *TATA box binding protein* (*Tbp*), *RPTOR independent companion of MTOR, complex 2* (*Rictor*), and *Ankyrin repeat domain-containing protein 27* (*Ankrd27*), as these are known to be unaffected in their expression levels during nerve injury and pain[18] (Fig. 2a). Next, we plotted genes that have been established to be overexpressed in DRG neurons in the presence of paclitaxel, including *Monocyte chemoattractant protein 1* (*Ccl2*). This gene mediates macrophage recruitment and promotes peripheral neuropathy in the presence of paclitaxel[19].

We further analysed the expression of *Itgb1*, which protects DRG neurons from paclitaxel-induced axon damage[20]. This revealed its upregulation, potentially a compensatory mechanism due to altered *Itgb1* trafficking in DRG neurons of paclitaxel-treated mice[20]. Since our research showed that increased MMP-13 activity in the epidermis promotes the development of paclitaxel-induced peripheral neuropathy, we further determined whether Mmp13 expression also changes in DRG neurons, which revealed no significant increase in these neurons.

To further validate skin-specific gene expression profiles, we choose to analyse the known keratinocyte housekeeping genes, *TATA box binding protein* (*Tbp), Ribosomal protein large P0* (*Rplp0*), and *Phosphoglycerate kinase 1* (*Pgk1*)[21,22], which as expected did not significantly vary in their expression levels regardless of treatment (Fig. 2b). To validate genes that we expected to display a change in expression, we first analysed *Mmp13*, which displayed a paclitaxel-dependent significant increase in expression in the skin, in line with our previous findings[2,8]. Next, we analysed the expression of *Tissue inhibitor of metalloproteinase-3* (*Timp3*), a known antagonist of MMP-13[23], which displayed a downward regulation trend, however this was not significant. Given the altered cell adhesion in the skin of paclitaxel-treated animals induced by increased MMP activity, we also analysed the tight junction protein, *Claudin 22* (*Cldn22*)[2,8]. Tight junctions, for instance, have been shown to be decreased upon MMP-13 activation in intestinal epithelia upon LPS stimulation[24]. Consistent with this finding, we detected a significant decrease in *Cldn22* expression in the skin[25] following paclitaxel treatment. This confirms that expected gene expression trends can be detected in our gene expression data sets, thus validating our approach. This data will be useful for the research community for further analysis.

Code Availability

The R code used to analyse and process the raw count data from skin and DRG samples using DESeq2 package version on R version 4.0.3 is publicly available at https://github.com/acirrincio/PaclitaxelRNAseq/.

Declarations

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Author contributions

These authors contributed equally: Anthony M. Cirrincione, Cassandra A. Reimonn.

These authors jointly supervised this work: Benjamin J. Harrison, Sandra Rieger.

Competing interests

The authors declare no competing interests.

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Table 1

Table 1 is available in the Supplementary Files section.

Figures

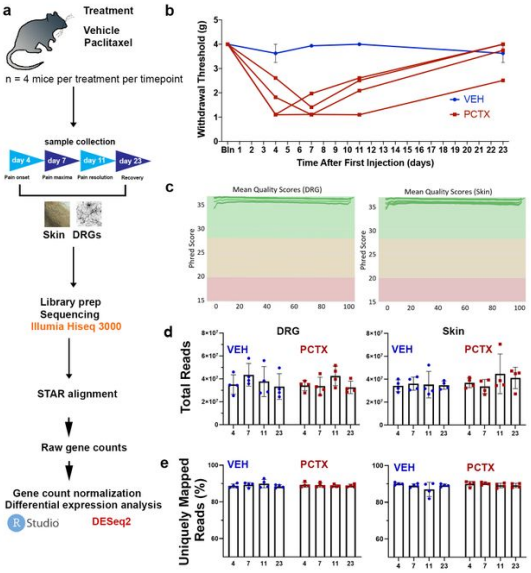

Figure 1

Experimental overview of longitudinal RNAseq analysis comparing dorsal root ganglion neurons and skin from paclitaxel and vehicle treated mice. (a) Summary of experimental workflow from treatment and sample collection to data trimming and analysis. (b) Behavioural analysis using von Frey filaments to assess the touch response showing the development of paclitaxel-induced neuropathy in mice evident by reduced pain threshold around 4, 7 and 11 days with a subsequent recovery (n=4 per treatment group per timepoint). (c) Read-quality scores for DRG neurons (left) and skin (right). (d, e) Total and uniquely mapped reads among DRG (left) and skin (right) samples from paclitaxel and vehicle treated animals.

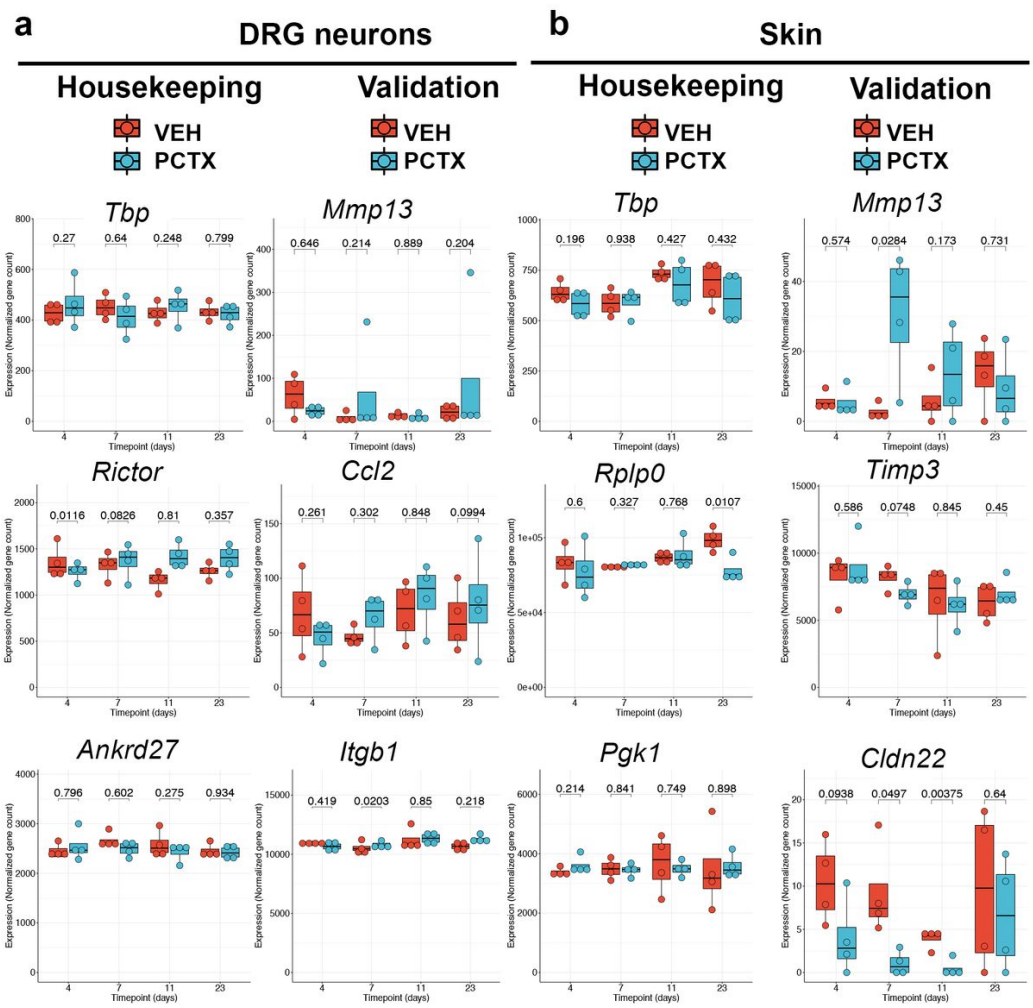

Figure 2

Technical validation of genes. Expression graphs of normalized gene counts showing housekeeping genes (no expected expression changes) and validation genes (expected expression changes) for (a) DRG neurons and (b) skin. (a) DRG validation genes include Ccl2 and Itgb1, known to be overexpressed in DRG neurons in the presence of paclitaxel, and Mmp13, only known to play a skin-specific role in paclitaxel-induced peripheral neuropathy. (b) Known housekeeping genes in skin include Tbp, RplpO, and Pgk1. Validation genes in skin include Mmp13, Timp3, a known inhibitor of MMP-13, and Cldn22, a tight junction protein in the epidermis. P-values from a two-sided t-test are represented above brackets for each timepoint.

Supplementary Files

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- Table1.xlsx