**Title:** The Molecular Signature Associated with Oxaliplatin Induced Peripheral Neuropathy in Colorectal Cancer

**Abstract**

**Background:** Oxaliplatin is the standard treatment option for colorectal cancer (CRC) which is one of the most prevalent forms of cancer. However, patients suffer either treatment discontinuation or adverse post-treatment life quality due to Oxaliplatin-induced peripheral neuropathy.

**Methods:** Our study has comprehensively explored the molecular mechanisms underlying Oxaliplatin-induced peripheral neuropathy via an extensive literature survey and identified multiple genes that may contribute to neuropathy and neurotoxicity. In addition to that, the publicly available bulk transcriptomic data of Illumina HiSeq 2500 platform, comprising the CRC tissue of 18 individuals' tumor and adjacent normal tissue was processed to identify differentially expressed genes (DEGs). Moreover, the single-cell RNA sequencing data of 10X genomics comprising normal and tumor tissues was subjected to analysis using Seurat and sctype R packages to uncover the cancer cells associated DEGs. Functional and pathway enrichment analysis was conducted using the Genecodis4 web-based tool. Next, RNA-seq data of CRC cell lines treated with Oxaliplatin compared with normal individuals, was also processed and DEGs were determined to validate the inhibition of a curated list of neuropathy-associated genes.

**Results:** From literature and database searches,a total of 1367 genes, including ion channel genes, normal sensory neuron-associated genes, and axon-excitability-related genes were collected that are either reported to be or may be contributing to neuropathy among cancer survivors upon oxaliplatin administration. The bulk transcriptomic data analysis revealed 715 DEGs and single-cell analysis uncovered 2,854 DEGs. Identified upregulated genes from single-cell data analysis, such as LGALS4, SPINK4, TFF3, REG4, and REG1A were found to be associated with tumor proliferation via epithelial-mesenchymal transitions, oxidative stress, dysregulated immune system, and inflammation which can be utilized as potential targets to devise novel therapeutic strategies for CRC treatment. Furthermore, many proteins involved in axon-excitability (NGF, SOD1, ROBO1, CNTNAP2, CNTNAP2, and KCNMB1), normal sensory neuron (SOX10, APOE, SST, S1PR1, and KCND3), voltage-gated sodium (SCNN1B, SCNN1G, SCNN1A, SCN1B, and SCN2B), calcium (CACNA2D2, CACNA1A, CACNA1C, CACNA1E, and CACNA1F), and potassium channels (KCND3, KCNMB1, KCNMA1, KCNJ2, and KCNN4) showed downregulation due to the oxaliplatin administration in CRC cell lines and their inhibition may have led to neuropathy which needs further validations.

**Conclusion**: Our study uncovers the downregulation of multiple genes upon oxaliplatin administration leading to neuropathy development and also elucidates potential therapeutic targets for better prognosis of CRC.

**Introduction**

Colorectal cancer (CRC), also known as bowel or colon cancer, develops in the colon or rectum, crucial parts of the large intestine responsible for nutrient absorption and waste removal [(1)](https://www.zotero.org/google-docs/?zCQld1). Initially, CRC often starts as benign polyps on the colon or rectum's inner lining, potentially becoming malignant over time [(2)](https://www.zotero.org/google-docs/?lztvS2). If left untreated, it can metastasize to other organs like the liver, lungs, or nearby lymph nodes [(3)](https://www.zotero.org/google-docs/?CU2Xgd). Common symptoms include changes in bowel habits, persistent abdominal pain, blood in the stool, unexplained weight loss, fatigue, and weakness [(4)](https://www.zotero.org/google-docs/?WUqr6r). In some cases, CRC may remain asymptomatic until reaching an advanced stage, underscoring the importance of regular screenings for early, more effective treatment [(5)](https://www.zotero.org/google-docs/?uPQP60).

It is a major public health concern being the second leading cause of cancer-related deaths and is the third most prevalent cancer worldwide [(6)](https://www.zotero.org/google-docs/?1pB7Yo). CRC has also been divided into two classes by the Cancer Genome Atlas (TCGA) project i.e., hypermutated tumors (16%) and non-hypermutated tumors (84%) [(7)](https://www.zotero.org/google-docs/?9Lw0YB). As per the GLOBOCAN statistics, the total new cases and the number of deaths estimated in 2020 were 1,931,590 and 935,173, respectively, across all ages in both sexes [(8)](https://www.zotero.org/google-docs/?eYa38i). The highest incidence and mortality rates of 52.3% and 54.2% were observed in Asia [(9)](https://www.zotero.org/google-docs/?o84EZS). The 5-year prevalence has been recorded in 5,253,335 persons across all populations [(10)](https://www.zotero.org/google-docs/?yJs2Yj). Furthermore, CRC is more common in men (23.4) than women (16.2) according to age-standardized incidence rates calculated per 100,000 persons [(11)](https://www.zotero.org/google-docs/?Qq6S77).

The onset of CRC is shaped by a range of risk factors, which encompass factors such as age, a family history of CRC or polyps, specific inherited genetic mutations, a prior history of inflammatory bowel disease (such as Crohn's disease or ulcerative colitis), the consumption of a diet high in red or processed meats, a sedentary way of life, obesity, smoking, and excessive alcohol consumption [(12)](https://www.zotero.org/google-docs/?QIGUmS).

Treatment for CRC varies based on factors such as cancer stage, location, and overall health [(13)](https://www.zotero.org/google-docs/?4lla0n). Options include surgery to remove tumors and nearby lymph nodes, radiation therapy, chemotherapy, targeted therapy, and immunotherapy. The primary goal is to eliminate or control cancer to prevent recurrence [(14)](https://www.zotero.org/google-docs/?Jx95nW).

However, these treatments have limitations, including drug toxicity, drug resistance, and aggressive CRC forms with metastasis [(15)](https://www.zotero.org/google-docs/?aDHL21). Common therapies like 5-fluorouracil (5-FU) combined with oxaliplatin or irinotecan, leucovorin, and monoclonal antibodies (e.g., Cetuximab, Bevacizumab) show reduced effectiveness in metastatic CRC due to acquired drug resistance [(16)](https://www.zotero.org/google-docs/?uzMfJh).

Oxaliplatin, a potent neurotoxic chemotherapy used in CRC treatment, forms DNA-platinum adducts within cancer cells, disrupting DNA replication and cell division [(17)](https://www.zotero.org/google-docs/?sjiTIX). While effective, it causes Oxaliplatin-induced peripheral neuropathy (OXAIPN), often requiring dose adjustments or interruptions due to its dose-limiting toxicity [(18)](https://www.zotero.org/google-docs/?DdNa7x). The OXAIPN is reported to be a common side effect with both acute and chronic manifestations; also, approximately 80% of the CRC patients treated with either oxaliplatin alone or with other combinational chemotherapeutics experience neurotoxicity [(19,20)](https://www.zotero.org/google-docs/?XMeD08). Moreover, in another study, it was reported for the first time that even after the shorter duration of the oxaliplatin (3 months) the oxaliplatin-induced peripheral neuropathy was still a pervasive issue [(21)](https://www.zotero.org/google-docs/?GhGx2z).

DNA-targeting drugs, crucial in cancer treatment, have drawbacks [(22)](https://www.zotero.org/google-docs/?SsUNA0). They lack specificity, affecting healthy cells and causing side effects like neurotoxicity, bone marrow suppression, and hair loss [(23)](https://www.zotero.org/google-docs/?PmUkMv). Resistance can develop, reducing efficacy [(24)](https://www.zotero.org/google-docs/?i92RQf). Treatment complexity, patient genetic variations, long-term effects, and high costs pose challenges for healthcare [(25)](https://www.zotero.org/google-docs/?YdW5LB).

OXAIPN primarily impacts sensory neurons, altering their function and causing various effects on neural activity, as well as alterations in ion channel activity and axonal excitability [(26)](https://www.zotero.org/google-docs/?HQYlxI). Oxaliplatin appears to affect the sodium channels in primary dorsal root ganglion (DRG) neurons, potentially playing a pivotal role in the development of neuropathic pain [(27)](https://www.zotero.org/google-docs/?yGFGO9). The proposed pathogenesis for chronic OXAIPN involves cellular metabolism decline, axoplasmic transport disruption, and apoptosis of DRG neurons due to the accumulation of oxalate in DRG cells, oxidative stress, and mitochondrial dysfunction [(28,29)](https://www.zotero.org/google-docs/?cELmLm).

Further side effects of Oxaliplatin include peripheral neuropathy, myelosuppression, gastrointestinal reactions, and allodynia [(30)](https://www.zotero.org/google-docs/?Lqz6WS). Treatment approaches include sodium channel blockers and targeting the Wnt3a/YTHDF1 pathway. Numerous genes, such as GSTPI, SCNA, KCNN3, ABCC2, AGXT, and others, are associated with OXAIPN, while sodium channel-related genes like SCN4A, SCN10A, and SCN9A, as well as proteins like OCT2, Caspase-3, GAP43, and pNfH, are linked to this condition, providing insights into its genetic and molecular underpinnings [(31,32)](https://www.zotero.org/google-docs/?1lGvsz). Its effects on the action potential's duration, the sodium current's peak, voltage–response relationship, inactivation current, and sensitivity to tetrodotoxin (TTX) [(33)](https://www.zotero.org/google-docs/?wpU7uu).

Considering these side effects and limitations of Oxaliplatin, understanding the mechanisms underlying OXAIPN is crucial for developing targeted interventions to mitigate its debilitating effects. Moreover, it is imperative that novel CRC therapeutic approaches are explored to counter the cancer progression and metastasis while making sure of the quality of life of the patient being administered. Therefore, to provide an alternative solution to highly toxic and off-target drugs, novel treatment approaches need to be explored against CRC, including targeted therapy, immunotherapy and natural products therapy. Also, no prior research has investigated the exploitation of Oxaliplatin in terms of off-targets at a larger scale. Several researchers have reported the use of off-target computational drug assessment techniques for drugs such as irinotecan, celecoxib, ofloxacin, and tamoxifen [(34–36)](https://www.zotero.org/google-docs/?uzR9D4).

Several studies have reported genes that are correlated to poor survival rate of CRC patients, such as HSP90, SLC2A3, PVT1, FOXD1, TIMP1, and KLK11 [(37–39)](https://www.zotero.org/google-docs/?Dt69jC). Further research on these genes can lead to targeted therapies for inhibiting CRC. Inhibition of these dysregulated genes can provide a potential and better solution for CRC treatment compared to Oxalipatin or other such toxic drugs by replacing them with Federal Drug Authority (FDA) approved drugs and polyphenols [(40–42)](https://www.zotero.org/google-docs/?p1SuLY).

Bulk and single-cell RNA-sequencing reveal crucial insights into CRC's molecular drivers, progression, and metastasis [(43,44)](https://www.zotero.org/google-docs/?davNJc). By examining dysregulated genes and their expression dynamics in cancer cells or tissues, we can identify key targets for inhibition to halt cancer advancement. This includes genes involved in cell proliferation, angiogenesis, immune evasion, and metastasis, shedding light on the tumor's aggressiveness and its potential to spread to other sites.

This study employs a comprehensive approach, consisting of an extensive literature review to investigate the genes associated with neuropathy resulting from Oxaliplatin administration and its impact on overall patient survival. The aim is to shed light on the molecular mechanisms underlying OXAIPN. Specifically, this research focuses on identifying the genes implicated in OXAIPN and assessing their potential inhibition by Oxaliplatin, which may contribute to neurotoxicity. Furthermore, this study utilizes bulk and single RNA-sequencing analysis pipelines to pinpoint significant molecular dysregulations that distinguish CRC patients from healthy individuals. The overarching objective is to uncover novel therapeutic targets that could be harnessed to ameliorate OXAIPN and enhance the treatment outcomes for colorectal cancer patients.

**Methodology**

In this study, we focused on chemotherapy-induced peripheral neuropathy (CIPN), which stands as the most prevalent form of neuropathy caused by drug treatments and exhibits substantial resistance to conventional analgesic interventions. The research encompassed an exhaustive review of existing literature and an exploration of cancer genomics. The primary objective of this study was to pinpoint the critical pathways and genes implicated in neuropathic conditions due to the use of oxaliplatin on CRC patients. Our research aimed to compile an extensive list of genes and proteins potentially contributing to neuropathy, with a particular emphasis on assessing the impact of their inhibition by oxaliplatin in terms of inducing neurodegeneration or neurotoxicity. Furthermore, our investigation involved the identification of overexpressed genes in CRC patients, employing both single-cell and bulk RNA-sequencing techniques. This analysis was conducted with the intent of uncovering novel therapeutic targets. These findings contribute to the broader endeavor of finding alternative treatment strategies for CRC beyond oxaliplatin.

**Curation of neuropathy-related genes list**

To identify the genes that are directly affected by the neurotoxic chemotherapeutic oxaliplatin, through literature review, various pathways were shortlisted to understand the mechanism of action of oxaliplatin and its role in peripheral neuropathy. The significant pathways were normal sensory neuron-related pathways, axonal excitability [(53)](https://www.zotero.org/google-docs/?v770Wq), and genes related to sodium, calcium, and potassium channels [(54,55)](https://www.zotero.org/google-docs/?jMAecn). Furthermore, genes via protein-protein interactions (PPI) within these pathways were also collected using the STRING database. STRING database systematically collects and integrates PPIs using automated text-mining techniques applied to scientific literature and computational interaction predictions [(56)](https://www.zotero.org/google-docs/?8UONLo).

**Genes retrieved from the literature**

Several genes associated with the normal sensory neuron were extracted from literature using PubMed and Google Scholar databases using the keyword search “normal sensory neuron.” Similarly, a list of genes associated with neurotoxicity caused by oxaliplatin and axon excitability-related genes was curated through PubMed and Google Scholar database searches using the keywords “neurotoxicity” and “axon excitability”.

Similarly, the genes related to worst survival and best survival in colorectal cancer were collected from published articles [(57)](https://www.zotero.org/google-docs/?RFk0fd) [(58)](https://www.zotero.org/google-docs/?zZiHYS) [(59)](https://www.zotero.org/google-docs/?PEE5pX) [(60)](https://www.zotero.org/google-docs/?BawfVK) [(61)](https://www.zotero.org/google-docs/?GCwUgS) using a similar approach of keyword search using keywords “worst survival colorectal cancer” “worst survival colon cancer,” “poor prognosis colorectal cancer,” “best survival colon” and “best survival colorectal cancer”. Furthermore, genes related to mismatch repair were included in the list reported to be dysregulated in CRC [(62)](https://www.zotero.org/google-docs/?SwuTNG)[(63)](https://www.zotero.org/google-docs/?3keTEM)**.**

**Genes retrieved from GeneCards**

All the genes related to ion channels, i.e., sodium, potassium, and calcium playing a role in membrane potential, were retrieved from GeneCards (<https://www.genecards.org/>) [(64)](https://www.zotero.org/google-docs/?QSOKeR). GeneCards is a user-friendly integrative database that allows researchers to collect comprehensive knowledge of all the predicted and annotated genes of humans [(64)](https://www.zotero.org/google-docs/?LNkY4F).

**Genes list curated from OMIM**

OMIM is an online catalogue of human genes and genetic disorders and stands for Online Mendelian Inheritance in Man (<https://www.omim.org/>) [(65)](https://www.zotero.org/google-docs/?PSwnDS). Axonal excitability-related genes were collected from the OMIM database, which provides comprehensive knowledge of the genes, their products, and phenotypes associated with those genes [(65)](https://www.zotero.org/google-docs/?x1Qqsd).

**Genes retrieved from REACTOME**

A list of genes playing a role in phototransduction, sensory perception of taste and hearing, and olfactory signaling pathways was retrieved from another database called REACTOME (<https://reactome.org/>) [(66)](https://www.zotero.org/google-docs/?Gc9Y15).

**Genes identified through network analysis**

Using the STRING database (<https://string-db.org/>) [(56)](https://www.zotero.org/google-docs/?JiCymx), protein-protein interactions (PPI) were performed to determine the networks of significant genes associated with oxaliplatin-induced peripheral neuropathy. STRING database aims to integrate all predicted and known associations between proteins based on physical interactions and functionality [(56)](https://www.zotero.org/google-docs/?j1KgCO). The genes, proteins, and sodium channels that are reportedly involved in peripheral neuropathy were shortlisted for the PPI using STRING. The genes associated with oxaliplatin-induced peripheral neuropathy include GSTP1, KCNN3, ABCC2, AGXT, CTR1, GSTM1, ERCC2, XRCC1, TAC1, FOXC1, ITGA1, ACYP2, DLEU7, BTG4, CAMK2N1, FARS2, CTSS, GSTM1, GSTT1, TRPA1 [(67–71)](https://www.zotero.org/google-docs/?ofeq0E). Sodium channels SCN4A, SCNA10A, and SCN9A are reportedly involved in neuropathy [(67)](https://www.zotero.org/google-docs/?Q1K5pK). Additionally, OCT2, Caspase-3, GAP43, and pNfH are the known proteins involved in causing peripheral neurotoxicity upon oxaliplatin administration [(72–74)](https://www.zotero.org/google-docs/?MPO11a).

**Single-Cell RNA-seq Analysis**

The study involved acquiring scRNA-seq data from three patients, yielding six samples (three from normal tissues and three from tumor tissues). The data was obtained from the NCBI GEO repository with accession ID GSE163974. The raw data underwent precise mapping using the STAR splice-aware aligner with specific parameters. This produced three distinct output files for each sample: a count matrix (in matrix market format), barcode identifiers, and gene identifiers. Quality control was performed using the Seurat 4.2.0 package [(75)](https://www.zotero.org/google-docs/?fAXWs4), evaluating metrics like library size, genes detected per barcode, and the proportion of UMIs mapping to mitochondrial genes. To account for technical variability, the "LogNormalize" method was applied for count normalization. Highly variable features (top 2000 genes) were identified, and the first 20 principal components (PCs) were computed to capture significant variation sources. These PCs were used for cell clustering and t-distributed stochastic neighbor embedding (t-SNE) visualization. An automated supervised clustering pipeline using the scType R package was employed for cell annotation, relying on marker genes from the "Immune System" database. Differential gene expression analysis was conducted to identify top marker genes for each cell type, offering insights into their expression patterns. Genes with differential expression patterns were then subjected to functional enrichment analysis, identifying enriched Gene Ontology terms and relevant KEGG pathways. This comprehensive annotation was performed using the enrichR package in R, shedding light on the biological implications of the identified differential gene expression patterns [(76)](https://www.zotero.org/google-docs/?X4Tu45).

**Bulk RNA-seq Analysis**

The study involved collecting paired-end RNA-sequencing data (GEO ID: GSE50760) from the NCBI GEO Datasets. This repository houses diverse microarray and high-throughput sequencing data for comparable samples with varying treatments. The data obtained from the Illumina HiSeq 2500 platform, comprised cDNA from the CRC tissue of 18 individuals' tumor and adjacent normal tissue. The preprocessing and analysis of RNA-seq data included quality assessment using FastQC, followed by filtration and decontamination using Fastp. Parameters such as -w (for processing speed), -i (for input reads), and -o (for output cleaned reads) were employed. The cleaned reads were then mapped to the reference human genome (GRCh38) using HISAT2 2.2.1, producing aligned reads in SAM format [(77)](https://www.zotero.org/google-docs/?DPMqi6). SAMtools functions view, and sort were used to convert SAM to BAM format and sort the aligned reads by chromosomal coordinates. To address duplicate reads (PCR artifacts), deduplication was performed using Sambamba 0.6.6 with the markdup function, with the -r flag used to remove marked duplicates. Subsequently, abundances of aligned reads were estimated using the reference-based transcriptome assembler, StringTie. This involved three key steps: transcript assembly, merging of annotated samples, and quantification of aligned reads. The resulting count files were then analyzed for differential gene expression using the Ballgown 2.30.0 package in R 4.2.2 [(78)](https://www.zotero.org/google-docs/?5Acuhc). While Ballgown identified biologically and statistically differentially expressed genes, significant genes were selected based on log2FC (indicating biological significance) and P-value (indicating statistical significance). Upregulated and downregulated genes were determined using the defined thresholds. Additionally, significantly expressed DEGs related to the disease (CRC) were validated through literature. This comprehensive analysis provided insights into the molecular characteristics associated with the studied condition.

**Validation of neuropathy-related genes inhibition by oxaliplatin**

To further validate our findings of inhibition of neuropathy-related genes by oxaliplatin, the transcriptomes of cell lines treated with oxaliplatin were compared with transcriptomes of adjacent normal colon and rectum tissues of CRC patients. To retrieve the transcriptomics data, the GEO public repository was employed, and two RNA sequencing datasets were utilized with accession IDs as PRJNA756841 and PRJNA802883 for oxaliplatin-treated cell lines and normal tissues, respectively. The data was preprocessed and analyzed using the tools and steps mentioned in the previous section of bulk RNA-seq analysis. However, for differential gene expression analysis between cell lines and colon and rectum tissue samples, the DESeq2 tool was used, which revealed significantly over-expressed and under-expressed genes with threshold of P-value < 0.05 and logFC > 1 and < -1, respectively.

Furthermore, the genes exhibiting downregulation due to oxaliplatin and also found in our curated genes set from literature and upregulated genes from RNA sequencing data analysis were identified using the Venny 2.0.2 web-based tool.

**Results**

**Neurotoxicity contributing genes catalog**

From literature and database searches, a total of 1367 genes were collected that are either reported to be or may be contributing to neuropathy among cancer survivors upon oxaliplatin administration. Among those, 210 genes related to axonal excitability were extracted from the OMIM database. Through the exploration of REACTOME, 641 significant genes have been identified and linked to phototransduction, sensory perception of taste and hearing, and olfactory signaling pathways. Moreover, 225 genes were identified from various experimental studies related to five categories, including normal sensory neurons, mismatch repair, and the best and worst overall survival in CRC patients having 49, 4, 68, and 104 genes, respectively. Channels-related genes, including sodium, calcium, and potassium channels, were extracted from GeneCards, having 21, 26, and 119 genes, respectively.

Furthermore, through STRING-based PPI analysis, 275 genes were identified and characterized in the context of their involvement in oxaliplatin-induced peripheral neuropathy. Based on literature evidence, the genes reported to be involved in neuropathy and neurotoxicity were shortlisted for PPI analysis to find other genes/proteins if dysregulated might contribute to neuropathy. The PPI analysis of GSTP1 included EPHX1, CYP2E1, CYP3A4, CYP1A1, MAPK8, PRDX6, CYP1B1, CYP1A2, GSR, and CYP2C9. AGXT gene network analysis via STRING showed that GRHPR, AGXT2, HOGA1, SHMT1, SHMT2, PSPH, PEX5, HAO1, KAT2B, and HAO2 are the directly interacting proteins. Moreover, CDK7, GTF2H4, MNAT1, GTF2H1, GTF2H2, GTF2E1, ERCC3, GTF2E2, MMS19, and XPA were found to show interaction with ERCC2.

Among the sodium channels that are distinctly involved in neurotoxicity, the SCN4A network analysis results showed that it directly interacts with SCN1B, CALM3, SCN4B, CLCN1, SCN3B, SCN2A, CALM2, SCN2B, SCNN1B, and SCNN1A. Additionally, SCN1B, SCN2B, SCN4B, SCN3B, SCLT1, TRPM4, SCN5A, S100A10, ANXA2, and ANK3 were found to be the interacting proteins of SCN10A protein. STRING-based network analysis revealed that SCN9A has direct interactions with SCN1B, SCN2B, SCN2A, SCN3A, CALHM1, SCN4B, GABRG2, NAV1, AGL, and MPV17.

The proteins reported to be involved in neuropathy include Oct2, caspase-3, GAP43, and NEFH, and their network analysis revealed that many significant proteins interact with them that may contribute to neuropathy in cancer patients upon oxaliplatin administration. The PPI results for OCT2 protein disclosed that SLC47A1, IGF2R, SLC47A2, EHMT2, SLC31A1, SLC6A2, YRDC, SLCO1B1, SLCO1B3, and RSC1A1 may interact with it directly. The details of all the genes, proteins, and their PPI interactions are provided in **Supplementary Sheet 1 Subsheet (Gene-Network Analysis),** and the figures for all these networks are provided in **Supplementary Document 2**. The details of each category of curated genes are depicted in **Table 3.4**. The gene names, their UniProt accession numbers, and functions are provided in the respective category-based sheets in **Supplementary Sheet 1**.

**Table 3.4. The number of genes curated that may play a role in causing neurotoxicity or neuropathy**

|  |  |  |
| --- | --- | --- |
| **Type** | **No. of Genes** | **Retrieval** |
| Expressed in normal sensory neuron | 49 | Literature |
| Related to phototransduction, sensory perception of taste and hearing, and olfactory signaling pathway | 641 | REACTOME |
| Playing a role in axonal excitability | 210 | OMIM |
| Related to sodium channel | 21 | GeneCards |
| Related to calcium channel | 26 | GeneCards |
| Related to potassium channel | 119 | GeneCards |
| Identified through network analysis | 275 | STRING |
| Related to poor overall survival in CRC | 104 | Literature |
| Related to best overall survival in CRC | 68 | Literature |
| Significant genes related to mismatch repair | 4 | Literature |
| Upregulated genes | 314 (7 were RNA and pseudogenes) | Differential gene expression analysis (DGEA) of CRC patients |

**Identification of differentially expressed genes via bulk RNA sequencing analysis**

Differential gene expression analysis revealed a total of 716 dysregulated genes in 36 CRC tumors vs normal samples (CRC tumor = 18, normal = 18). A total of 314 upregulated genes were observed with a fold change (FC) value > 1 and P-value < 0.05, while 401 downregulated genes with an FC value <-1 and P-value < 0.05 in CRC.

The dysregulated genes concerning biological and statistical significance are demonstrated in **Figure 3.1 (A)**. In contrast, the top 10 upregulated and downregulated genes in terms of log FC are shown in **Supplementary Document 4 Table S1,** and **Table S2**, respectively. The top 10 upregulated genes included MMP7, DPEP1, CLDN1, SPP1, ALB, APOA2, HP, CXCL8, THBS2, and H19, and the top 10 downregulated genes included CLCA4, CA1, AQP8, CA7, GUCA2B, GUCA2A, CEACAM7, CLCA4, CA4, and OTOP2.

**GO term analysis of dysregulated genes**

To identify GO term of dysregulated genes, GeneCodis4 was performed that identified the biological processes (BP), Cellular Component (CC), and Molecular Functions (MF) that were affected by CRC. The BP analysis demonstrated that upregulated genes were enhanced in negative regulation of endopeptidase activity, lipid metabolic process, chemotaxis, inflammatory response, neutrophil chemotaxis, proteolysis, negative regulation of peptidase activity, positive regulation of heterotypic cell-cell adhesion, negative regulation of peptidase activity, angiogenesis and lipoprotein metabolic process **(Supplementary Document 4 Figure S1, Table S3)**. Nevertheless, the downregulated genes were enriched in immunoglobulin production, immune response, adaptive immune response, positive regulation of B cell activation, phagocytosis-recognition, complement activation, classical pathway, B cell receptor signaling pathway, phagocytosis engulfment, and proteolysis **(Supplementary Document 4 Figure S2, Table S4)**.

The MF analysis demonstrated that upregulated genes play a role in serine-type endopeptidase activity, serine-type endopeptidase inhibitor activity, cytokine activity, CXCR chemokine receptor binding, heparin-binding, extracellular matrix structural constituent, endopeptidase inhibitor activity, growth factor activity, peptidase inhibitor activity, and serine-type peptidase activity **(Supplementary Document 4 Figure S3, Table S5)**. While the downregulated genes were involved in antigen binding, immunoglobulin receptor binding, extracellular matrix structural constituent, hormone activity, galactoside binding, carbonate dehydratase activity, hexosyl transferase activity, peptidase activity, guanylate cyclase activator activity, and symporter activity **(Supplementary Document 4 Figure S4, Table S6)**.

The CC analysis of upregulated genes revealed that they were majorly localized in the extracellular region, collagen-containing extracellular matrix, blood microparticle, extracellular exosome, endoplasmic reticulum lumen, extracellular matrix, high-density lipoprotein particle, platelet alpha granule, and collagen trimer **(Supplementary Document 4 Figure S5, Table S7)**. In contrast, downregulated genes were mostly located in the immunoglobulin complex, collagen-containing extracellular matrix, extracellular exosome, immunoglobulin complex, circulating plasma membrane, apical plasma membrane, secretory granule, peptidase complex, microfibril, and basolateral plasma membrane **(Supplementary Document 4 Figure S6, Table S8)**.

**KEGG pathway analysis of dysregulated genes**

Dysregulated genes interrupted the biological pathways, which were identified through GeneCodis4. The upregulated genes were involved in overexpressed biological pathways such as complement and coagulation cascades, IL-17 signaling pathway, rheumatoid arthritis, amoebiasis, alcoholic liver disease, TNF signaling pathway, bile secretion, PPAR signaling pathway, cytokine-cytokine receptor interaction, and biosynthesis of amino acids **(Supplementary Document 4 Figure S7, Table S9)**. While downregulated genes were underexpressed in bile secretion, proximal tubule bicarbonate reclamation, nitrogen metabolism, pentose and glucuronate interconversions, renin-angiotensin system, mineral absorption, protein digestion and absorption, pancreatic secretion, neuroactive ligand-receptor interaction, and aldosterone-regulated sodium reabsorption **(Supplementary Document 4 Figure S8, Table S10)**.

**Single-Cell RNA Sequencing Analysis of CRC**

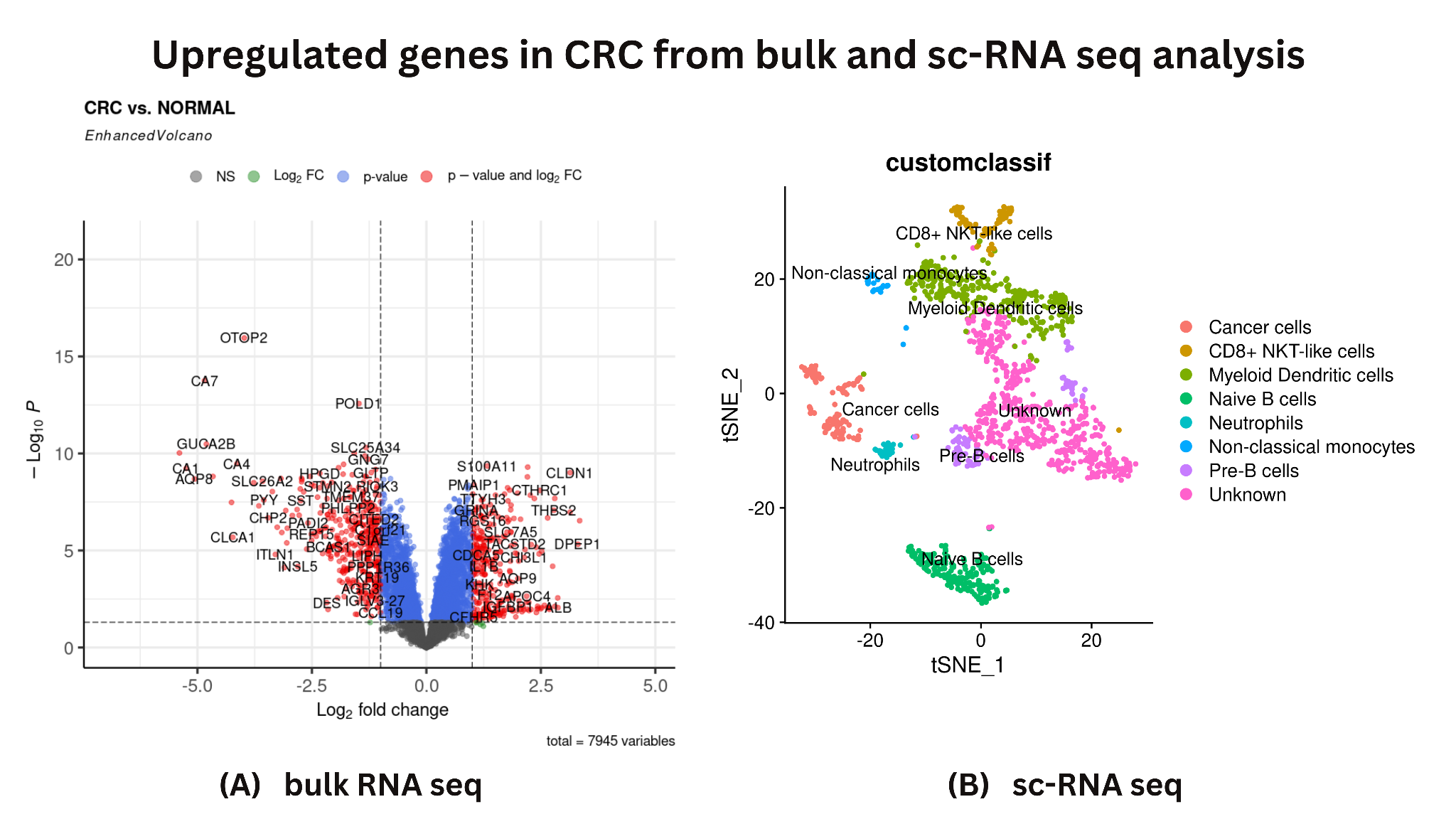
The scRNA-seq data analysis of the samples revealed eight cell types (Cancer cells, CD8+ NKT-like cells, Myeloid Dendritic cells, Naive B cells, Neutrophils, Non-classical monocytes, Pre-B cells, and Unknown cells) in our dataset. Among these cell types, the unknown cells were excluded from the data as their genes did not show a specific expression for annotation that could lead to uncertain results. Furthermore, the remaining seven cell types (Cancer cells, CD8+ NKT-like cells, Myeloid Dendritic cells, Naive B cells, Neutrophils, Non-classical monocytes, and Pre-B cells) showed 13,998 differentially expressed genes in their cells. Out of all these genes, 7,838 genes were upregulated, and 6,160 genes were found to be downregulated. Moreover, the genes unique to cancer cells were compared with the genes representing all the cell types; consequently, it was found that there were a total of 2,854 dysregulated genes in the cancer cells (1,635 genes were upregulated, and 1,219 genes were downregulated).

Additionally, the top five upregulated and downregulated genes were found for all the cell types. The top five dysregulated (upregulated and downregulated) genes for Naive B cells were IGHG2, IGHG3, IGHG1, IGLC2, and IGHA1 (upregulated), while ACTG1, ACTB, TMSB4X, SPINK4, and S100A6 (downregulated), for Myeloid dendritic cells, MS4A1, CD37, CD74, SMIM14, and LTB (upregulated), while IGHG3, IGHA1, IGHG1, IGHG2, and JCHAIN (downregulated), for CD8+ NKT-like cells, CD3D, IL32, GZMA, CCL5, and GNLY (upregulated), while IGHG1, IGHG3, IGHG2, IGLC3, and IGLC2 (downregulated), for Pre-B cells, H2AZ1, TUBB, TUBA1B, HMGB2, and H4C3 (upregulated), while AGR2, SPINK4, TFF3, IGHG3, and IGHA1 (downregulated), for Non-classical monocytes, S100A9, S100A8, DCN, CFD, and IGFBP6 (upregulated), while IGHA1, IGKC, IGLC2, IGLC3, and IGHG1 (downregulated), for Neutrophils, LCN2

LYZ, CSTB, S100A6, and PRSS2 (upregulated), while JCHAIN, IGHA1, IGKC, IGLC3, and IGLC2 (downregulated). Lastly, the top five upregulated genes for cancer cells were found to be LGALS4, SPINK4, TFF3, REG4, and REG1A, while its downregulated genes were IGHA1, IGKC, IGLC2, IGLC3, and IGHG2, respectively. The number of upregulated and downregulated genes identified by sc-RNA sequencing analysis are shown in **Table 3.3**. The identified significant marker genes in eight different cell types are shown in **Figure 3.1 (B)**. The heatmap displaying all the dysregulated marker genes of all cell types, including cancer cells, is depicted in **Figure 3.2**. The expression values of all the differentially expressed marker genes in all cell types are provided in **Supplementary Sheet 6**.

**Table 3.3.** Single-cell RNA sequencing analysis of CRC showing the total number of genes in the dataset, total upregulated and downregulated genes, and cancer cells differentially expressed genes compared to other cell types in the dataset.

|  |  |
| --- | --- |
| Total differentially expressed genes in the dataset | 13,998 |
| Total upregulated genes in the dataset | 7,838 |
| Total downregulated genes in the dataset | 6,160 |
| Total cancer cells' differentially expressed genes | 2,854 |
| Cancer cells upregulated genes | 1,635 |
| Cancer cells down-regulated genes | 1,219 |



**Figure 3.1**. **(A)** Enhanced volcano plot representing significant differentially expressed genes in CRC. Red dots represent upregulated and downregulated genes. Biologically significant genes are shown on the x-axis w.r.t Log2FC ± 1, and statistically significant genes are shown on the y-axis w.r.t Log10 P-value < 0.05.

**(B)** Cell subpopulation analysis revealed significant marker genes in eight different cell types identified via sc-RNA sequencing analysis of CRC patients. Different colors depict different cell types, including cancer cells.



**Figure 3.2.** Heatmap showing the significantly dysregulated marker genes of each cell type.

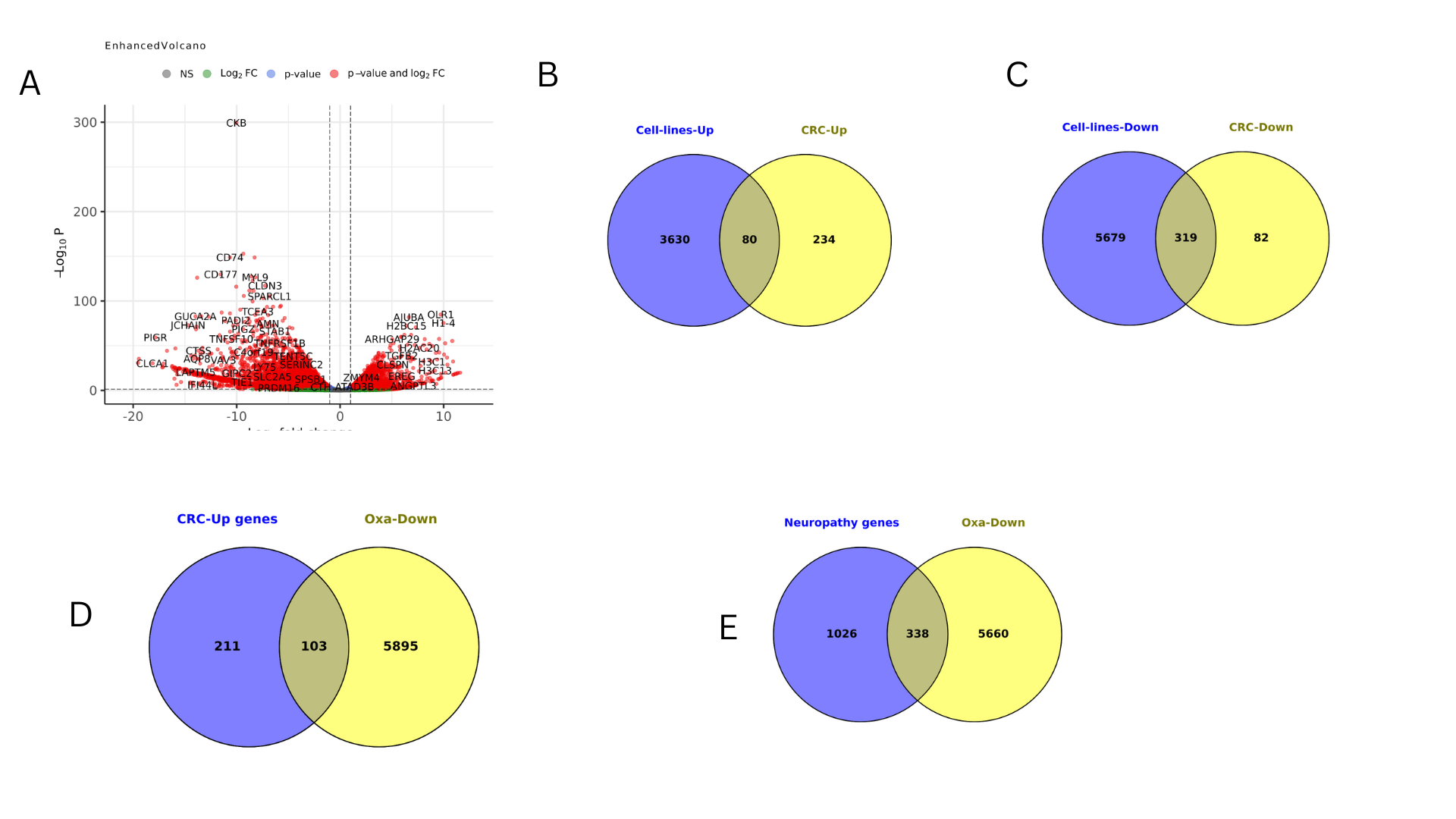
**Pathway enrichment analysis of dysregulated markers of cancer cells**

The Reactome pathway analysis of the upregulated markers of the cancer cells revealed the overexpressed biological pathways such as metabolism, respiratory electron transport, ATP synthesis by chemiosmotic coupling, heat production by uncoupling proteins, citric acid (TCA) cycle and respiratory electron transport, respiratory electron transport, complex I biogenesis, cellular responses to stimuli, asparagine N-linked glycosylation, metabolism of proteins, translation, and cellular responses to stress (**Supplementary Document 5 Figure S7, Table S7**). However, the downregulated markers indicated the pathways such as cell cycle, cell cycle, mitotic, immune system, signaling by Rho GTPase, signaling by Rho GTPases, miro GTPases and RHOBTB3, adaptive immune system, mitotic metaphase and anaphase, RHO GTPase effectors, mitotic anaphase, and M phase (**Supplementary Document 5 Figure S8, Table S8**).

**Validation of inhibition of neuropathy-related genes**

The differential gene expression analysis revealed that 3710 genes were upregulated, meeting the threshold of logFC > 1 and 5998 genes showed under-expression with logFC < -1. The DEGs related to biological and statistical significance are depicted in **Figure 3.3 (A)**. Moreover, the common genes identification disclosed that 80 upregulated genes and 319 downregulated genes were common between the two datasets, including the bulk-RNA seq dataset of CRC vs normal patients and oxaliplatin-treated cell lines dataset, shown in **Figure 3.3 (B)** and **(C)**. The top upregulated genes with high expression levels that showed upregulation even after oxaliplatin administration were found to be KLK6, OLR1, KRT80, MSX2, and CLDN2.

However, when the upregulated genes in CRC and the curated list of genes associated with neuropathy were compared with the downregulated genes of oxaliplatin-treated cell lines identified for validation, 103 and 338 genes were found to be overlapping, shown as Venn diagrams in **Figure 3.3 (D)** and **(E)**. All the aforementioned common genes are provided in the **Supplementary Sheet**. Among these 338 downregulated genes, the genes belonging to each category were extracted to pinpoint which genes' downregulation may contribute to oxaliplatin-induced peripheral neuropathy (**Supplementary Files**).



**Figure 3.3**: Validation of inhibition of upregulated genes in CRC via oxaliplatin.

**Discussion**

Chemotherapeutic drug infusion, for instance, oxaliplatin, often leads to side effects, including neuropathy and neurotoxicity, limiting the patient‘s health during and after cancer treatment. Oxaliplatin, a platinum-based drug that plays a central role in first and second-line therapies for CRC, is usually administered with a fluorouracil/leucovorin-based regimen [(79)](https://www.zotero.org/google-docs/?q8g8Yg). Its adverse effects can develop as an acute syndrome during or shortly after treatment or gradual development of chronic sensory neuropathy over time, limiting the patient’s health during and after cancer treatment [(80)](https://www.zotero.org/google-docs/?W8MMbX). Oxaliplatin has been reportedly known to form DNA adducts by interacting with the nucleus and mitochondria within the neuron cells that subsequently lead to the impairment of vital processes such as replication and transcription and contribute to severe neurotoxicity by effectively accumulating in the dorsal root ganglia (DRG), the region where sensory neurons are present [(81)](https://www.zotero.org/google-docs/?QwCYdh). Several genes, including multiple drug transporters such as OCT2, MATE1, CTR1 and others, have been reported to mediate the platinum accumulation of drugs in DRG neurons [(81)](https://www.zotero.org/google-docs/?274C40).

Recent studies have shown that cancer patients experienced oxaliplatin-induced neuropathy-related symptoms soon after oxaliplatin administration, affecting about 85 to 95% of all patients [(82)](https://www.zotero.org/google-docs/?tDqmkS). Hence, there exists a necessity for a more profound understanding of potential off-target interactions of oxaliplatin prior to its binding with the intended DNA Dodecamer Duplex target.

The present study has unraveled various genes and proteins through an exhaustive literature review that may contribute to neuropathy and neurotoxicity by oxaliplatin administration. A total of 1367 genes from different databases and published studies have been curated belonging to different categories, including expressed in normal sensory neurons, related to phototransduction, sensory perception of taste and hearing, and olfactory signaling pathway, playing a role in axonal excitability, related to sodium channel, related to calcium channel, related to potassium channel, related to poor overall survival in CRC, related to best overall survival in CRC, and significant genes related to mismatch repair.

Among this curated gene list, sodium channels SCN4A, SCNA10A, and SCN9A are reportedly involved in neuropathy [(67)](https://www.zotero.org/google-docs/?c9dm7U). Additionally, the genes associated with oxaliplatin-induced peripheral neuropathy include GSTP1, KCNN3, ABCC2, AGXT, CTR1, GSTM1, ERCC2, XRCC1, TAC1, FOXC1, ITGA1, ACYP2, DLEU7, BTG4, CAMK2N1, FARS2, CTSS, GSTM1, GSTT1, TRPA1 [(67–71)](https://www.zotero.org/google-docs/?VvKDsJ). Notably, OCT2, Caspase-3, GAP43, and pNfH are the known proteins involved in causing peripheral neurotoxicity upon oxaliplatin administration. These genes and proteins were subjected to PPI analysis to further elucidate which downstream genes and proteins may be affected by these neurotoxicity-associated genes and whether they contribute to neuropathy.

Next, to identify which neurotoxicity-associated genes can potentially be inhibited by oxaliplatin administration, the transcriptome of CRC cell lines treated with oxaliplatin was compared with the curated list of genes. When compared with the downregulated genes of oxaliplatin-treated cell lines, 338 neuropathy-associated genes were observed to overlap among them. These findings suggest that these genes may potentially be inhibited by oxaliplatin which leads to neuropathy and neurotoxicity in CRC survivors. Among these common genes, majorly potassium (KCNA6, KCNAB2, KCNE4, KCNF1, KCNG1, KCNH2, KCNIP1, KCNJ12, KCNJ15, KCNJ16, KCNK5, KCNK6, KCNN3, KCTD12, KCTD18, KCTD5, KCTD7, and more), sodium (SCN4A, SCN7A, SCN11A, and SCN3B), and calcium channel proteins (CACNA1A, CACNA1C, CACNA1E, CACNA1F and so on) and other sensory neuron-related genes, including some axon excitability-related genes whose inhibition may significantly cause neurotoxicity and peripheral neuropathic pain in CRC patients and survivors.

Voltage-gated sodium ion channels (Nav) are known to be the most significant channels associated with OXAIPN [(55)](https://www.zotero.org/google-docs/?Zic6fN). The first literature evidence in support of this argument was reported in a study performed on mice’s hippocampus neurons, dorsal root ganglia, and sensory nerve preparations. The results showed an increase in the amplitude of action potentials in A-fibres of rat sural and vagal nerves upon oxaliplatin administration, indicating that oxaliplatin modulates Nav [(83)](https://www.zotero.org/google-docs/?HSJMnh). calcium channels are known to be the key mediators involved in neuron sensitization [(84)](https://www.zotero.org/google-docs/?KNCv9e). They play a critical role in neurotransmitter secretion by increasing the local calcium concentration in the pre-synaptic cell. P/Q-type calcium channels have been reported to link the neuronal excitation in pre-synaptic neurons for secreting neurotransmitters [(85)](https://www.zotero.org/google-docs/?Mm8991); hence, their inhibition via oxaliplatin may disrupt neurotransmission.

Furthermore, in the next phase of this study, bulk and single-RNA sequencing pipelines were employed to identify the genes whose over-expression significantly contributes to the progression and development of CRC and how these genes lead to dysregulation of multiple cellular biological processes and functional pathways.

Among the top significantly overexpressed genes identified via bulk transcriptomics, MMP7 is reportedly known to be associated with tumor invasion and, thereby shows metastatic potential [(86)](https://www.zotero.org/google-docs/?ptoUzG). Similarly, DPEP1 upregulation is reported in the early stages of colon carcinogenesis, leading to tumor invasion [(87)](https://www.zotero.org/google-docs/?2IbBZg). CLDN1 in CRC is a well-known prognostic marker that is majorly responsible for the intercellular barrier function of tight junctions. Higher expression of CLDN1 in CRC tissues as compared to normal has been linked to malignant behaviour and the worst overall survival of the patient [(88)](https://www.zotero.org/google-docs/?11bV34).

Intriguingly, single-cell transcriptomics revealed some top overexpressed genes identified in cancer cells that are known to be tumor-associated, including SPINK4, TFF3, REG4, and REG1A. These genes contribute to CRC progression and development as SPINK4 promotes CRC proliferation via enhanced expression [(89)](https://www.zotero.org/google-docs/?h4Hu7O), TFF3 activates the EMT process to contribute to metastasis in CRC [(90)](https://www.zotero.org/google-docs/?kwHKGI), and tumor migration and invasion was found to be promoted by overexpression of REG4 [(91)](https://www.zotero.org/google-docs/?2wWKg2), and poor-prognosis was uncovered in early stage CRC patients due to upregulated REG1A [(92)](https://www.zotero.org/google-docs/?JX0OAo).

The upregulated genes from the bulk-RNA sequencing analysis were found to be mainly involved in the pathways such as lipid metabolic process, inflammatory response, neutrophil chemotaxis, Acyl-CoA metabolic process, proteolysis, and angiogenesis. However, the top significant upregulated genes identified via sc-RNA sequencing analysis were observed to be involved in metabolism, respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins. All these pathways are known to be major contributing factors in cancer progression and development [(93–96)](https://www.zotero.org/google-docs/?sPPBza) and also affect the prognosis.

Moreover, the administration of oxaliplatin may not effectively inhibit the overexpressed genes that contribute to CRC development and progression. To identify the biomarkers whose over-expression leads to CRC even after oxaliplatin administration, this study has uncovered multiple genes (**Supplementary Sheet**) using bulk RNA-seq analyses of CRC patients as well as cell lines treated with oxaliplatin. The upregulated genes, which overlapped between the transcriptomes of CRC patients and the oxaliplatin-treated cell lines, were determined to pinpoint the biomarkers that may be utilized as therapeutic agents for CRC treatment, as oxaliplatin failed to inhibit them.

Furthermore, the genes identified through sc-RNA seq analysis which may significantly contribute to tumorigenesis, could be used as potential therapeutic biomarkers for CRC treatment using drugs and compounds other than oxaliplatin.

It is, therefore, suggested to validate these findings of inhibition of multiple genes via oxaliplatin, subsequently leading to neurotoxicity and also experimentally and bioinformatically assess the over-expressed therapeutic biomarkers determined from sc-RNA seq analysis for better therapeutic strategies against CRC.

**Conclusion**

Oxaliplatin-induced peripheral neuropathy makes colorectal cancer treatment quite devastating despite being the standard. This study has identified various neuropathy-associated oxaliplatin off-target proteins related to normal sensory neurons, axon excitability, and voltage-gated sodium, potassium, and calcium channels, which are also validated further if they show upregulation in the presence of oxaliplatin. Next, the bulk and single-cell transcriptomics analysis revealed significant CRC therapeutic targets that are majorly involved in tumor proliferation, invasion, and worst survival, such as LGALS4, SPINK4, TFF3, REG4, and REG1A. Hence, inhibiting proteins involved in inflammation, tumor proliferation, and invasion via drugs other than oxaliplatin with no neurotoxic adverse effects would be an effective CRC treatment. However, oxaliplatin off-targets and therapeutic targets uncovered through this comprehensive bioinformatics study should be validated experimentally to be practiced as treatment.

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