Chapter 6

Understanding regulatory mechanism of anticancer potential of CdS NPs using transcriptomics studies.

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6.1 Background-

Transcriptomics, a field within the realm of molecular biology, offers a comprehensive understanding of gene expression patterns and regulatory mechanisms within cells. The advent of high-throughput sequencing technologies has revolutionized transcriptomics studies, enabling researchers to investigate the dynamic interplay of genes in response to various stimuli, including treatment with plant therapeutics. This burgeoning area of research holds immense promise for elucidating the molecular mechanisms underlying the therapeutic effects of plant-derived compounds on cellular processes.

Plant therapeutics have long been revered for their medicinal properties, with traditional herbal remedies serving as the foundation for modern drug discovery. These natural compounds contain a myriad of bioactive molecules that can modulate cellular pathways involved in health and disease. Transcriptomics studies seek to unravel the intricate network of gene expression changes induced by plant therapeutics, shedding light on their molecular targets and mechanisms of action.

Cell lines serve as invaluable model systems for transcriptomics studies, offering a controlled environment to dissect the effects of plant therapeutics on gene expression profiles. By treating cell lines with various plant-derived compounds or extracts, researchers can probe the transcriptome to identify differentially regulated genes implicated in therapeutic responses. These genes may encode proteins involved in diverse cellular processes, including signal transduction, metabolism, immune response, and cell proliferation.

The utilization of next-generation sequencing (NGS) technologies, such as RNA-sequencing (RNA-seq), has revolutionized transcriptomics research by enabling the comprehensive profiling of gene expression at a genome-wide scale. RNA-seq facilitates the quantification of transcript abundance, splice variants, and novel transcripts, providing unprecedented insights into transcriptional dynamics. Upon treatment with plant therapeutics, RNA-seq analysis allows for the identification of genes that are upregulated or downregulated compared to untreated control cells.

One of the primary objectives of transcriptomics studies is to decipher the molecular pathways modulated by plant therapeutics. By analyzing the differentially expressed genes (DEGs) in treated cell lines, researchers can gain insights into the biological processes affected by the therapeutic intervention. Pathway analysis tools, such as gene ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, facilitate the interpretation of transcriptomic data by highlighting the functional categories and pathways enriched with DEGs. These analyses unveil the cellular processes targeted by plant therapeutics, providing mechanistic insights into their pharmacological effects.

Moreover, transcriptomics studies enable the identification of key transcription factors (TFs) and regulatory elements orchestrating the cellular response to plant therapeutics. TFs play a central role in gene regulation by binding to specific DNA sequences and modulating the expression of target genes. By integrating transcriptomic data with TF binding site prediction algorithms, researchers can unravel the regulatory networks governing the transcriptional response to plant-derived compounds. This systems-level approach elucidates the transcriptional regulatory circuits activated or repressed upon treatment, offering a deeper understanding of the molecular mechanisms underlying therapeutic efficacy.

In addition to elucidating the direct effects of plant therapeutics on gene expression, transcriptomics studies can uncover secondary responses and crosstalk between signaling pathways. For example, treatment with a plant-derived compound may trigger a cascade of gene expression changes through the activation of upstream signaling pathways or the modulation of transcriptional regulators. By examining the temporal dynamics of gene expression and network interactions, researchers can unravel the intricate web of molecular events triggered by plant therapeutics.

6.2. Introduction

RNA-Sequencing (RNA-seq) is a valuable tool that compares the gene expression levels of normal and cancerous cells and facilitates the development of new therapeutic agents for cancer treatment.[191] RNA-Seq analysis has shown potential in identifying new biomarkers for Cancer by analysing gene expression changes during different stages and levels of the disease. This technology compares gene expression between drug-treated and non-treated cell lines, to determine which genes are up- or down-regulated[192].

Differential expression analysis, a key aspect of RNA-Seq data analysis, enables the investigation of altered cellular processes between biological functions through gene ontology (GO) analysis[193]. Additionally, the analysis can provide insights into enriched biological pathways using data from the Kyoto Encyclopedia of Genes and Genomes (KEGG)[194]. KEGG is a bioinformatics tool that analyse gene function, molecular interaction and link genomic data (GENE database) with functional information[195]. Differential expression analysis and RNA-Seq data analysis techniques are frequently applied in drug development studies and cancer research, including ovarian cancer, lung cancer, prostate cancer, bladder cancer, colorectal cancer, and gastric cancer.[196][197]

Cervical Cancer is an epithelial tumor, that strikes fear into the hearts of women worldwide as the second most common female cancer, posing a dangerous threat to their health and ranking high as a leading cause of cancer death[198]. Its incidence in young women has increased from 10% to 40% over the past three decades[199]. Global estimates show that 85% of cancer deaths occur in developing countries, which is 18 times higher than in developed countries[200]. At present, surgery, radiotherapy, and chemotherapy are the commonly used treatment approaches for cancer[201]. To date, substantial progress in the treatment of cancer has been limited due to the limited understanding of the molecular alterations that occur at different stages of the disease.

The metal-based nanoparticles or quantum dots (QD) have garnered substantial interest in the field of oncology for tumor imaging, drug delivery, and cancer cell destruction. [202]Quantum dots exhibit reactive oxygen species generation capacity and possess the advantage of small size (typically <10 nm)[203]. Cadmium sulphide QD has been recognized as a promising candidate for cancer cell treatments due to favourable physicochemical characteristics, low cytotoxicity, and biocompatibility[204]. The utilization of plant extracts as stabilizing agents in the synthesis of CdS quantum dots is a promising approach for the control of quantum dot size while ensuring low toxicity, and cost-effectiveness. It is suitable for in vitro studies, as it has been shown to not cause significant cellular damage[205][206]. Recent research has shown tremendous potential for usage in *invivo* bioimaging and cancer therapeutics using *Camellia sinensis* extract as a stabilizing agent for CdS quantum dots.[207]

In this study, *Berberis aristata* (*Daruharidra*) extract was used as the particle stabilizing agent in the manufacture of CdS nanoparticles. The ayurvedic plant *Berberis aristata* is used to cure a variety of ailments, including hepatitis, malaria, jaundice, fever, bleeding, and diarrhea. [208]. It is a hardy, yellow, and spiny erect shrub commonly found in the sub-Himalayan regions.[209] *Daruharidra* is composed of various significant phytochemicals including alkaloids of the

proto-berberine and isoquinoline type, as well as bis benzyl-isoquinoline and flavonoids and phenolic acids which play an important role in Cadmium sulfide quantum dots synthesis[210]. We present a novel application of RNA-sequencing and transcriptomic analysis to identify gene targets and insight into molecular alterations in the Cancer cell line upon treatment with Cadmium sulfide nanoparticles derived from *Berberis aristate* extract. Furthermore, we conducted an analysis of the gene ontology and KEGG pathways linked with differentially expressed genes.

6.3. Methodology-

6.3.1 Total RNA Extraction, Qualitative and Quantitative analysis

The PA1 Cancer cell lines were obtained from the NCCS in Pune, India. The cells were kept alive by being cultivated in a regulated setting at 37°C with 5% CO_2 in a dedicated incubator. Fetal bovine serum (FBS) at a 10% supplement was added to the growth medium, which was DMEM. Every other day, the cell culture media had to be changed as part of routine maintenance in order to supply vital nutrients and preserve a favourable growth environment. After thawing, cell passaging was also restricted to 10 passages in order to protect the integrity of the cell line and prevent possible genetic changes.

Cadmium sulfide nanoparticles (CdS NPs), which were introduced to the cells at the start of the experimental phase, were then given to them at a concentration that was set at the IC_{50} value, or the concentration that inhibits 50% of cell growth. This was carried out to examine the impact of CdS NPs on the Cancer cell line within the given circumstances.

Using the Zymo Research Quick RNA MiniPrep Plus Kit and following the manufacturer's instructions, total RNA extraction was completed. Two techniques were used to evaluate the quality and quantity of the RNA samples. The absorbance of the RNA samples was first measured using the NanoDrop spectrophotometer, which offers details on the RNA's

concentration and purity. To further assess the RNA's integrity and quality, an Agilent TapeStation system with High Sensitivity RNA ScreenTape was used.

Overall, the Cancer cell culture was established and maintained, the effects of CdS NPs were investigated, and the quantity and quality of the isolated RNA samples were carefully measured in preparation for further molecular analysis.

6.3.2 Nano drop analyses-

The concentration of the RNA samples was determined using the NanoDrop spectrophotometer (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, NC, USA) for quantitative analysis. A range of 2 to 3000 ng/L is the range of RNA concentrations that the NanoDrop instrument can measure. After correcting the pH of the solution, the absorbance ratios (260/280 and 260/230) were measured in order to evaluate the purity of the RNA.

An approximate 260/280 and 260/230 ratio of 2 is considered indicative of "pure" Illumina 2x150 PE library preparation. The RNA samples that cleared quality control were used to create the RNA-Seq paired-end sequencing libraries. The NEBNext® UltraTM II Directional RNA Library Prep Kit for Illumina (NEB) was used to prepare the libraries in accordance with the manufacturer's instructions. The library preparation process for transcriptomics studies involves several crucial steps to ensure accurate and robust sequencing results. Firstly, mRNA is enriched from the total RNA sample using magnetic beads with Poly-T attachment. This step selectively captures mRNA molecules, allowing for a more focused analysis of protein-coding transcripts. Next, the enriched mRNA undergoes enzymatic fragmentation to break it into smaller fragments, which facilitates the subsequent steps of RNA-dependent synthesis. The fragmented mRNA serves as a template for the synthesis of the first-strand cDNA using NEBNext First Strand Synthesis Enzyme Mix. This enzyme mix catalyzes the reverse

transcription of mRNA into complementary DNA (cDNA), preserving the information encoded in the RNA molecules.

Following the synthesis of the first-strand cDNA, the second strand of cDNA is synthesized using a second-strand mix, generating double-stranded cDNA molecules. Subsequently, the double-stranded cDNA is purified using AMPure XP beads, which selectively bind and remove contaminants such as primers, nucleotides, and enzymes. This purification step ensures the removal of impurities that could interfere with downstream sequencing reactions.

To prepare the cDNA fragments for sequencing, A-tailing and adapter ligation are performed. A-tailing involves the addition of adenine nucleotides to the 3' ends of the cDNA fragments, which provides a template for adapter ligation. Adapter sequences containing specific barcodes and sequencing priming sites are ligated to the A-tailed cDNA fragments, allowing for the identification of individual samples and priming of the sequencing reactions.

Finally, the library is enriched by performing a limited number of PCR cycles to amplify the adapter-ligated cDNA fragments. This amplification step increases the quantity of DNA fragments available for sequencing while minimizing bias introduced during PCR amplification. The enriched library is then ready for quality assessment and sequencing on a next-generation sequencing platform, enabling comprehensive analysis of gene expression profiles in the transcriptome. Overall, each step in the library preparation process plays a critical role in ensuring the generation of high-quality sequencing data for transcriptomics studies.

These steps were carefully executed to ensure the preparation of high-quality RNA-Seq libraries suitable for Illumina sequencing, allowing for further downstream analysis of gene expression and other molecular studies.

6.3.3 Quantity and quality check (QC) of library on Agilent 4200 Tape Station

After the PCR enrichment step, the libraries were purified using AMPure XP beads and subjected to analysis on the 4200 Tape Station system (Agilent Technologies) with high sensitivity D1000 Screen tape. The purpose of this analysis was to evaluate the quality and quantity of the libraries, ensuring they meet the required standards for Next-Generation Sequencing (NGS).

To determine if the extracted RNA possessed the necessary quality and quantity for successful NGS, we followed the guidelines provided by the TruSeqVR RNA Access Library Preparation Kit from Illumina (Illumina, San Diego, CA, USA, catalogue number: RS-301-2001). These guidelines serve as a benchmark to ensure that the RNA libraries are prepared to the appropriate specifications, enabling accurate and reliable NGS results.

6.3.4 Cluster Generation and Sequencing

Once the mean peak sizes from the Agilent Tape Station profile and the Qubit concentration for the libraries were obtained, the Paired-End (PE) Illumina libraries were put onto the NextSeq500 platform for cluster building and sequencing. The PE sequencing approach allows template fragments to be sequenced in both forward and reverse orientations using the NextSeq500 equipment.

Complementary adapter oligos on a paired-end flow cell were coupled to the samples using kit reagents as part of the library preparation procedure. These adapters were specifically designed to enable cleavage of the forward strands only upon the resynthesis of the reverse strand during sequencing. Consequently, the cloned reverse strand, which was utilized to sequence from the opposite end of the fragment, allowed the genetic information contained in it to be fully read

out in both directions. This PE sequencing strategy enhances the accuracy and completeness of the sequencing data obtained from the NextSeq500 platform.

Functional Annotation and Pathway Analysis:

Gene Annotation: The first step in understanding the biological relevance of differentially expressed genes is to annotate them with functional information. This involves assigning gene ontology (GO) terms, which describe the biological processes, cellular components, and molecular functions associated with each gene. Additionally, genes can be annotated with information about known biological pathways in which they participate. This annotation process provides insight into the potential roles of the genes in various cellular processes and pathways.

Pathway Analysis: Once genes are annotated, pathway analysis tools and databases such as KEGG, Reactome, or Gene Ontology are utilized to investigate enriched pathways and biological processes associated with the differentially regulated genes. Pathway analysis helps identify the interconnected network of genes involved in specific biological pathways or processes. By examining the enrichment of differentially expressed genes within these pathways, researchers can gain insights into the underlying molecular mechanisms driving the observed changes in gene expression.

6.4. Results and Discussion

To understand the anti-cancerous molecular mechanisms within Cancer cells treated with CdS NPS. A detailed transcriptome analysis was done of the Cancer cells without and with the treatment of CdS NPs for 48 hours in triplicates. For the analysis, RNA quality assessment and sequencing were carried out.

6.4.1 RNA Quality and Quantity Assessment

To assess the integrity of the isolated RNA samples, we performed QC analysis using NanoDrop followed by Agilent TapeStation using High Sensitivity RNA ScreenTape. Both the

samples, Control and treated, obtained RIN (RNA Integrity Number) scores of 8.6 and 8.1 refer to Figure 6.1, respectively. RIN scores indicate the RNA quality on a scale of 1-10, with 10 being the highest integrity of the extracted RNA and vice versa.

It has been universally settled that the acceptable RIN level for samples with high inter-granularity ought to range around 7-8.[211], [212] Henceforth, our data is above the threshold RIN score. RIN scores above 8 are considered to be high-quality, intact RNA samples, between 5 to 8 moderately damaged RNA samples, and below 5 poorly damaged RNA samples.[213] As per electropherograms in Figure 6.1(b), 18S and 28S rRNA subunits are prominently identified as high-quality intact subunits. And these peaks are comparable with the control peak intensities.

6.4.2 RNA sequencing and identification of DEGs in Cancer cell line treated with CdS NP

Sequencing of the control and CdS NPS libraries produced 14 and 13 million raw reads, or around 4.24 and 3.61 Gb of data for each of them, respectively. To brief, 7.85 Gb of raw RNA reads was collected altogether, 4.24 Gb of which corresponded to the untreated group (Control, triplicates), whereas 3.61 Gb from the treatment group (CdS NPs treated, triplicates). By removing adapter sequences, ambiguous reads (reads with unknown nucleotides This sequenced raw data for samples was processed to create high-quality clean reads (reads with more than 10% quality threshold (QV) 25 phred scores) and low-quality sequences (reads with "N" higher than 5%). A total of 5.42 Gb of high-quality reads of RNA data combining both samples were obtained, out of which 3.17 Gb came from the control group and 2.25 Gb from the treatment group (CdS NPs).

The primary objective of the investigation is to recognize DEGs between untreated and CdS NPs-treated cancer cell lines and discover novel genes associated with the regulatory mechanisms of cancer. In control vs. CdS NPs, we found 35352 DEGs (Differentially

expressed genes), of which 1641 genes had substantially regulated DEGs with padj<0.001. As per the earlier reported criteria [214] with log2FoldChange >2 and padj<0.001, 1588 genes determined a significant upregulated DEG. We identified 53 significantly downregulated genes applying padj<0.001 and log2FC<-2.

6.4.2.1 Volcano plot

The genes are shown as dots in the volcano plot in Figure 6.2, combined with red dots showing upregulated DEGs with log FC>2 and padj<0.001, green dots signifying downregulated DEGs with padj<0.001 and log FC<-2, and yellow dots indicating insignificant accountability. The y-axis represents the average gene expression in terms of the (negative) value of log10 p-value, while the x-axis shows the DEGs' log2 fold change. This graphic indicates that only genes with a high average normalized count—the previously indicated threshold criteria—contain enough data to provide a significant conclusion.[214] The spots that don't meet the logFC and p-value criteria are shown as yellow dots in the graph as insignificant conclusions.

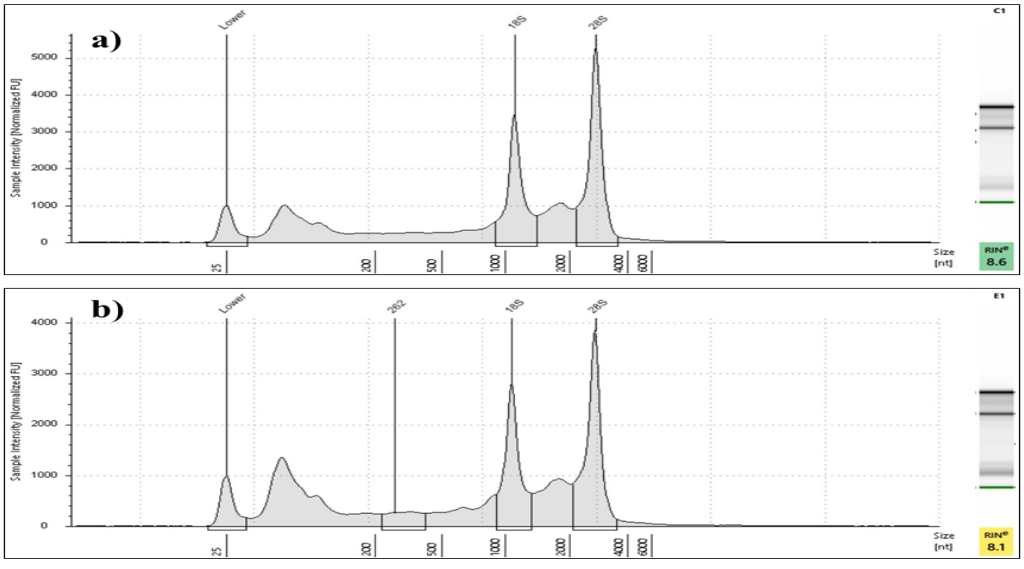


Figure 6.1: QC of RNA sample, on Agilent Tape station: a) electropherograms of Control b) electropherograms of CdS NPs

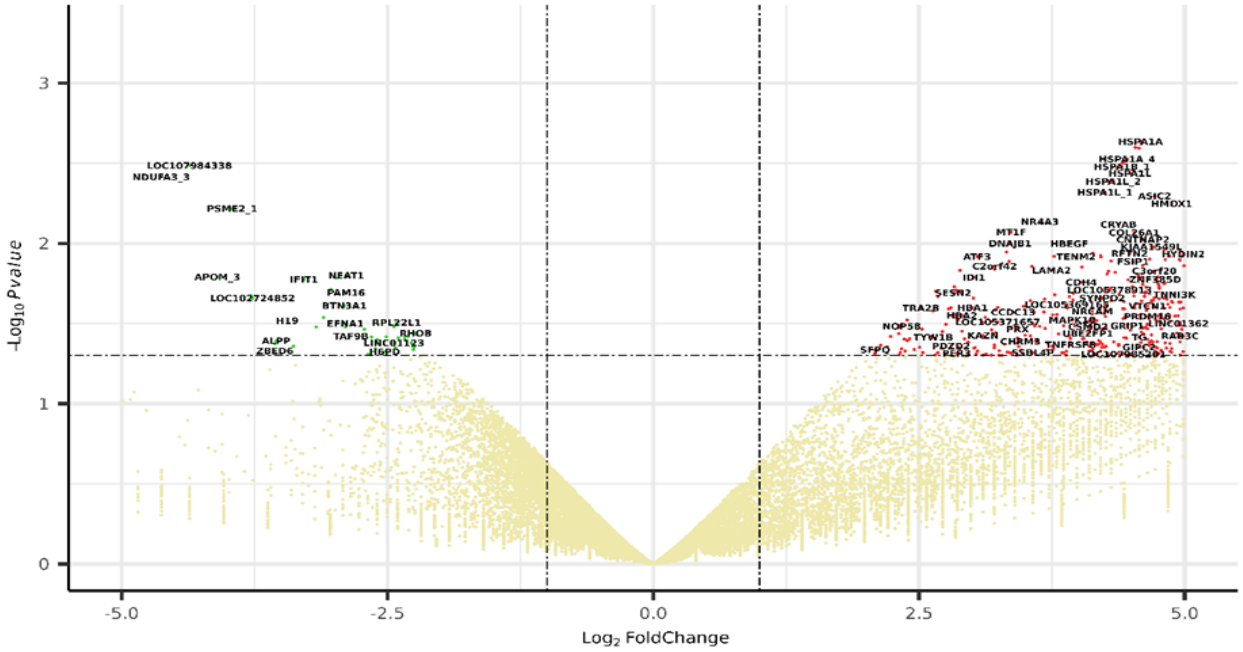


Figure 6.2: Volcano plot of the DEGs observed in CdS NPs treated cells: Red colored dots showing upregulated DEGs with log FC>2, green dots signifying downregulated DEGs with log FC<-2, whereas yellow dots indicating insignificant accountability

As per Figure 6.2, the differentially expressed genes were decoded for their roles in the apoptotic pathways. The complete list of the significant DEGs as per the volcano graph, with their previously reported functions (https://www.genome.jp/kegg/genes.html, https://www.ncbi.nlm.nih.gov/gene/) is represented in Table 1 and Table 2. Upregulated DEGs as per volcano includes: HSPA1A, HSPA1A 4, HSPA1B1, HSPA1L2, HSPA1L, HSPA1L_1, ASIC2, HMOX1, NR4A3, CRYAB, MT1F, COL26A1, DNAJB1, HBEGF, CNTNAP2, and, ATF3. Downregulated DEGs include: NDUFA3_3, PSME2_1, APOM-3, IFIT1, NEAT1, PAM16, BTN3A1, H19, EFNA1, ALPP, TAF9B, RHOB, RPL22L1, ZBED6, H6PD, and, LINC01123.

In order to retain the equilibrium of biological proteins, a broad range of chaperones identified as heat shock proteins (HSPs) are recognized to be essential. These have a variety of functions,

ranging from apoptosis, senescence, autophagy, immunological action, etc. HSPs, which in certain cases, govern proteostasis while exhibiting anti-apoptotic properties. Significant levels of HSPA2 correspond with poor clinical survival, but a contrary relationship has been identified for HSPA1, making HSPs an immunotherapeutic-drug target for investigating new chemotherapeutic medicines.[215]

Similarly, other upregulated and downregulated genes that have importance to cancer signalling or apoptotic signalling are listed below. ASIC2 stimulates Colorectal cell lines' division, growth, invasion, and metastasis both in vitro and in vivo. Extensive biochemical research, such as the ChIP-seq method, revealed that pursuant to acidosis, ASIC2 promotes the calcineurin/NFAT1 (nuclear factor of activated T cells) signaling cascade in which NFAT1 interacts to genes grouped in the networks for Rho GTPase signaling and calcium signaling. One of the primary pathways that precede calcium signalling is calcineurin/NFAT. Numerous studies have demonstrated the importance of NFAT factors in the development and advancement of tumors, including tumor expansion, migration, and vasculature.[216] Another gene HMOX1 gene, is classified to have ferroptosis-inducing properties in endothelium cells by increasing iron surplus intake, ROS production, and lipid peroxide.[217]

The expression of HMOX1 was increased in parthenolide-treated human thyroid BCPAP cancer cells.[218] Similar ROS-mediated apoptosis was reported in A549 lung cancer-treated cells reported with elevated NR4A3 levels.[219] Other upregulated genes include CRYAB, which is generally considered a prognostic marker for a variety of different cancer types. As a molecular chaperon, its overexpression is because it promotes cell integrity by blocking protein degradation resulting in tumor growth progression.[220] In addition, the details of the different other DEGs are listed in Tables 6.1 and 6.2.

Table 6.1: Significantly upregulated genes as per volcano graph and proposed function obtained from KEGG and NCBI database

S NO	Significantly upregulated genes	Pathways
1	HSPA1A, HSPA1A 4, HSPA1B1, HSPA1L2, HSPA1L, HSPA1L_1 (Heat shock protein family A (Hsp70))	MAPK signalling pathway, Antigen processing and presentation, Endocytosis, Spliceosome, Protein processing in the endoplasmic reticulum.
2	ASIC2 (Acid-sensing ion channel 2)	Taste transduction, Inflammatory mediator regulation of TRP channels, Ligand-gated channels, Acid-sensing ion channel
3	HMOX1 (Heme oxygenase 1)	Metabolic pathways, Pathways in cancer, HIF-1 signalling pathway, Chemical carcinogenesis - reactive oxygen species, Ferroptosis, Hepatocellular carcinoma
4	NR4A3 (Nuclear receptor subfamily 4 group A member 3)	Transcriptional misregulation in cancer, Protein families: signalling and cellular processes
5	CRYAB (Crystallin alpha B)	Protein processing in the endoplasmic reticulum
6	MT1F (Metallothionein 1F)	Mineral absorption, Messenger RNA biogenesis
7	COL26A1 (Collagen type XXVI alpha 1 chain)	Protein digestion and absorption, Protein families: signalling and cellular processes
8	DNAJB1 (DnaJ homolog subfamily B member 1)	Protein processing in the endoplasmic reticulum, Genetic Information Processing, Folding, sorting, and degradation
9	HBEGF (Heparin-binding EGF-like growth factor)	ErbB signalling pathway, GnRH signalling pathway, Proteoglycans in cancer

10	CNTNAP2 (Contactin associated protein-like 2)	Cell adhesion molecules, Signalling molecules, and interaction
11	ATF3 (Activating transcription factor 3)	Protein families: genetic information processing

Table 6.2: Significantly downregulated genes as per volcano graph and proposed function obtained from KEGG and NCBI database

S. No	**Significantly Downregulated genes**	**Pathways**
1	NDUFA3_3 (NADH: ubiquinone oxidoreductase subunit A3)	Metabolic pathways, Chemical carcinogenesis - reactive oxygen species, Oxidative phosphorylation, Thermogenesis
2	PSME2_1 (Proteasome activator subunit 2)	Proteasome, Antigen processing, and presentation
3	APOM-3 (Apolipoprotein M isoform X3)	Unclassified: signalling and cellular processes
4	IFIT1 (Interferon-induced protein with tetratricopeptide repeats 1)	Interferon signalling
5	NEAT1 (Nuclear paraspeckle assembly transcript 1)	transcriptional regulator for numerous genes, including some genes involved in cancer progression
6	PAM16 (Presequence translocase associated motor 16)	Protein families: signalling and cellular processes, Eukaryotic cytoskeleton proteins, Actin filaments / Microfilaments
7	BTN3A1 (Butyrophilin subfamily 3 member A1)	Protein families: signalling and cellular processes, Antigen processing and presentation
8	H19	long non-coding RNA, which functions as a tumor suppressor
9	EFNA1 (Ephrin A1)	MAPK signalling pathway, Ras signalling pathway, PI3K-Akt signalling pathway, MicroRNAs in cancer, GF-RTK-RAS-ERK signalling pathway

10	ALPP (Alkaline phosphatase, placental)	Metabolic pathways, Folate biosynthesis, Thiamine metabolism, Biosynthesis of cofactors
11	TAF9B (TATA-box binding protein associated factor 9b)	Basal transcription factors
12	RHOB (Ras homolog gene family, member B)	Biomarker of breast cancer, Protein families: signalling and cellular processes, cellular response to hydrogen peroxide; cellular response to ionizing radiation; and regulation of cell migration
13	RPL22L1 (Ribosomal protein L22 like 1)	cytoplasmic translation, Genetic Information Processing
14	ZBED6 (Zinc finger BED-type containing 6)	Transcription factors
15	H6PD (Hexose-6-phosphate dehydrogenase/glucose 1-dehydrogenase)	Pentose phosphate pathway, Metabolic pathways, Steroid hormone biosynthesis, Carbon metabolism
16	LINC01123 (Long intergenic non-protein coding RNA 1123)	Cancer proliferation: signalling

6.4.3 GO (Gene Ontology) Enrichment Analysis of DEGs

Figure 6.3 shows the functional aspects of the significantly enriched genes. Here we have briefly highlighted some of them that can be thought of as playing an important role in cancer progression or cancer cell apoptosis. One such is C3HC4-type RING finger domain GO, which generally acts as a DNA-binding motif in transcription factor TFIIIA. Also involved in several processes involving transcription, mRNA trafficking, signal transduction, and ubiquitination by allowing binding with protein, DNA, and lipids.[221], [222], [223] Other significantly enriched GO's are composed of ion channel activity, Transporter complex, and calcium ion transport; enrichment of these GO's necessary for coordinating upstream and downstream signalling involved in the cell cycle regulation of Cancer cells.[224], [225]

Positive regulation of protein tyrosine kinase activity (GO:0050731) is generally required to rate up any process requiring peptidyl tyrosine phosphorylation, which is generally helpful in cellular signal transduction. These are involved in tumor cell proliferation, metastasis, and Epithelial-mesenchymal transition (EMT) signalling and thus can be used as a potential target for targeted cancer therapy.[226]

Further, in order to better comprehend, we further examined the resulting significant DEGs for GO enrichment analysis. The analysis was done via enrichR; all three main biological activities: were biological processes (BP), cellular components (CC), and molecular functions (MF). Figure 6.4 and Figure 6.5 highlight the upregulated and downregulated GO, respectively, in terms of BP, CC, and MF. The classification is composed of the major significant DEGs having padj<0.001.

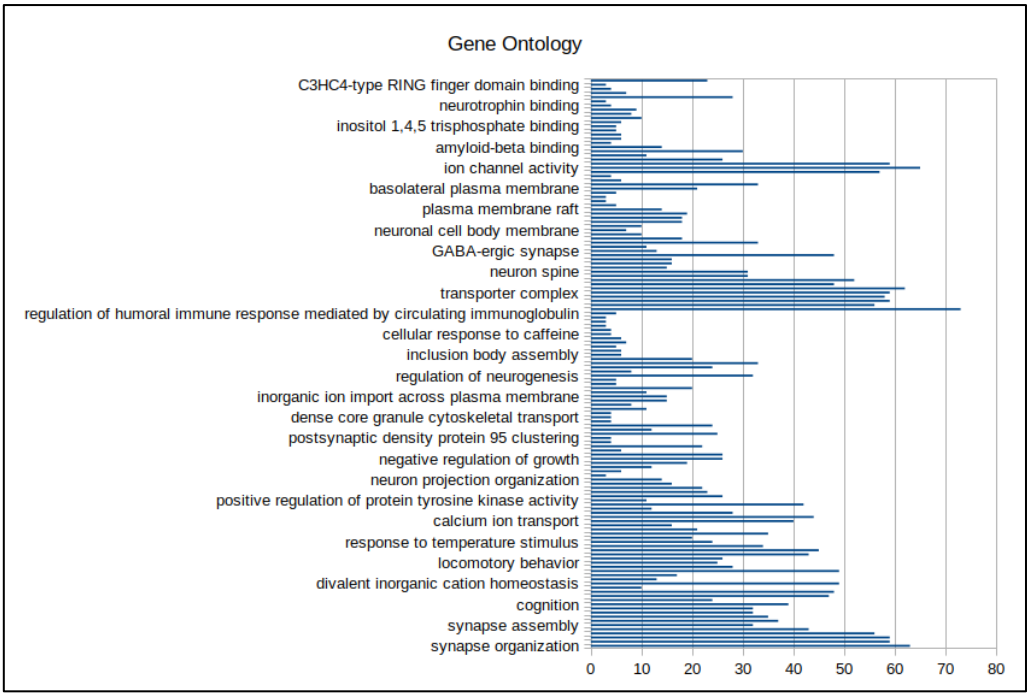
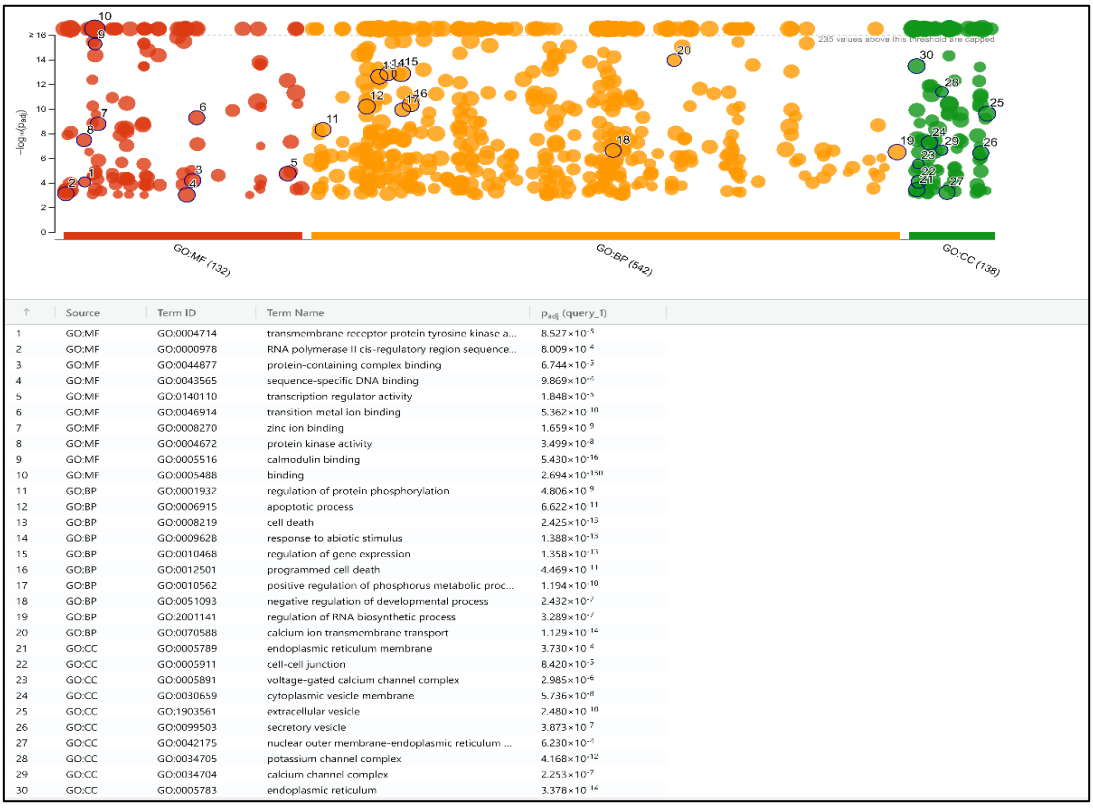


Figure 6.3: Gene Ontology of the significant DEGs upon Cancer cells treated with CdS NPs



	Source	Term ID	Term Name	P_{adj} (query_1)
1	GO:MF	GO:0004714	transmembrane receptor protein tyrosine kinase a...	8.527×10^{-5}
2	GO:MF	GO:0000978	RNA polymerase II cis-regulatory region sequence...	8.009×10^{-4}
3	GO:MF	GO:0044877	protein-containing complex binding	6.744×10^{-3}
4	GO:MF	GO:0043565	sequence-specific DNA binding	9.869×10^{-4}
5	GO:MF	GO:0140110	transcription regulator activity	1.848×10^{-3}
6	GO:MF	GO:0046914	transition metal ion binding	5.362×10^{-10}
7	GO:MF	GO:0008270	zinc ion binding	1.659×10^{-9}
8	GO:MF	GO:0004672	protein kinase activity	3.499×10^{-8}
9	GO:MF	GO:0005516	calmodulin binding	5.430×10^{-16}
10	GO:MF	GO:0005488	binding	2.694×10^{-150}
11	GO:BP	GO:0001932	regulation of protein phosphorylation	4.806×10^{-9}
12	GO:BP	GO:0006915	apoptotic process	6.622×10^{-11}
13	GO:BP	GO:0008219	cell death	2.425×10^{-13}
14	GO:BP	GO:0009628	response to abiotic stimulus	1.388×10^{-13}
15	GO:BP	GO:0010468	regulation of gene expression	1.358×10^{-13}
16	GO:BP	GO:0012501	programmed cell death	4.469×10^{-11}
17	GO:BP	GO:0010562	positive regulation of phosphorus metabolic proc...	1.194×10^{-10}
18	GO:BP	GO:0051093	negative regulation of developmental process	2.432×10^{-7}
19	GO:BP	GO:2001141	regulation of RNA biosynthetic process	3.289×10^{-7}
20	GO:BP	GO:0070588	calcium ion transmembrane transport	1.129×10^{-12}
21	GO:CC	GO:0005789	endoplasmic reticulum membrane	3.730×10^{-4}
22	GO:CC	GO:0005911	cell-cell junction	8.420×10^{-3}
23	GO:CC	GO:0005891	voltage-gated calcium channel complex	2.985×10^{-6}
24	GO:CC	GO:0030659	cytoplasmic vesicle membrane	5.736×10^{-8}
25	GO:CC	GO:1903561	extracellular vesicle	2.480×10^{-10}
26	GO:CC	GO:0099503	secretory vesicle	3.873×10^{-7}
27	GO:CC	GO:0042175	nuclear outer membrane-endoplasmic reticulum ...	6.230×10^{-4}
28	GO:CC	GO:0034705	potassium channel complex	4.168×10^{-12}
29	GO:CC	GO:0034704	calcium channel complex	2.253×10^{-7}
30	GO:CC	GO:0005783	endoplasmic reticulum	3.378×10^{-14}

Figure 6.4: GO of the upregulated DEGs showing Molecular function (MF) in red, Biological processes (BP) in yellow and cellular components (CC) in green.

Many pivotal genes and corresponding pathways associated with Cancer cell line were elucidated in the work. Initially, we got 35352 DEGs, with 1641 significantly expressed genes, of which 1588 upregulated and 53 downregulated significant DEGs were identified between control and treated. Next, we obtained categorical GO for the significant DEGs; out of 1588 upregulated genes, 812 GO-enriched terms (threshold padj<0.001) were obtained. In Figure 6.4, the categorical GO terms can be localized with 132 (MF), (542 BP), and 138 (CC) GO terms.

The significant mentions include (GO:0006915) apoptotic process and (GO:0012501) programmed cell death, enrichment of both these GO results in the apoptotic activity of the CDS NPS on Cancer cells. Other noteworthy processes include (GO:0000978) RNA polymerase II, (GO:0140110) transcription regulator activity, (GO:0008219) cell death, (GO:0009628) response to abiotic stimulus, (GO:0051093) negative regulation of developmental processes, (GO:1903561) extracellular vesicle, (GO:0070588) calcium ion transmembrane transport. Calcium signalling has been reported to have cancer cell proliferative and metastasis activity [227], [228], in addition to the Ca^{+}-dependent apoptotic pathway.[229] Extracellular vesicles are one of the major hallmarks of cancer cells and can be a potent therapeutic target because they are generally specific to a cell type.[230] In a nutshell, the GO upregulated terms, almost each GO terms in Figure 6.4, correspond to the cell signalling and gene regulation processes, which may be overexpressed due to cancer stage proliferation and to the later effect of apoptotic changes after treatment.

We obtained 35 GO-enriched terms (padj<0.001) out of 53 DEGs for the categorical GO as per the downregulated genes. Refer to Figure 6.5; the categorical GO includes 5 (MF), 17 (BP), and 13 (CC). Noteworthy, Go-enriched down DEG terms are (GO:0003674) molecular function, (GO:0010468) regulation of gene expression, (GO:0010605) negative regulation of macromolecule metabolic process, (GO:0065007) biological regulation. The gene IFIT1 has

been shown to enhance the epithelial-mesenchymal transition (EMT) of pancreatic cancer cells and to control Wnt/-catenin signaling, promoting cancer growth.[231] The downregulation of IFIT1 suggests negative regulation of cancer cell growth. All the downregulated GO mechanism ensures that the cell's basic pathways undergo negative regulation, thus reacting to the apoptotic conditions caused due to CdS NPS treatment.

ID	Source	Term ID	Term Name	p_{adj} (query_1)
1	GO:MF	GO:0005515	protein binding	2.708×10^{-8}
2	GO:MF	GO:0003674	molecular_function	3.330×10^{-7}
3	GO:MF	GO:0097159	organic cyclic compound binding	2.362×10^{-4}
4	GO:BP	GO:0006950	response to stress	4.091×10^{-4}
5	GO:BP	GO:0010468	regulation of gene expression	2.323×10^{-3}
6	GO:BP	GO:0010467	gene expression	5.449×10^{-3}
7	GO:BP	GO:0010605	negative regulation of macromolecule metabolic p...	5.692×10^{-4}
8	GO:BP	GO:0048519	negative regulation of biological process	6.528×10^{-3}
9	GO:BP	GO:0065007	biological regulation	7.945×10^{-7}
10	GO:CC	GO:0005575	cellular_component	1.233×10^{-8}
11	GO:CC	GO:0005634	nucleus	1.878×10^{-4}
12	GO:CC	GO:0043231	intracellular membrane-bounded organelle	5.044×10^{-5}

Figure 6.5: GO of the downregulated DEGs showing Molecular function (MF) in red, biological processes (BP) in yellow and cellular components (CC) in green.

6.4.4 KEGG analysis of DEGs

The expression statistics for each gene have been mapped to the KEGG pathway in order to determine if the DEGs are enabled or inhibited in an independent set of pathways. Making use of enrichR software, pathway modelling and functional characteristics of up as well as downregulated genes were retrieved. To comprehensively elucidate the KEGG pathway, 812

upregulated and 35 downregulated DEGs were identified in total ($p<0.001$). Table 6.3 lists the top 10 enriched routes (UP and DOWN)

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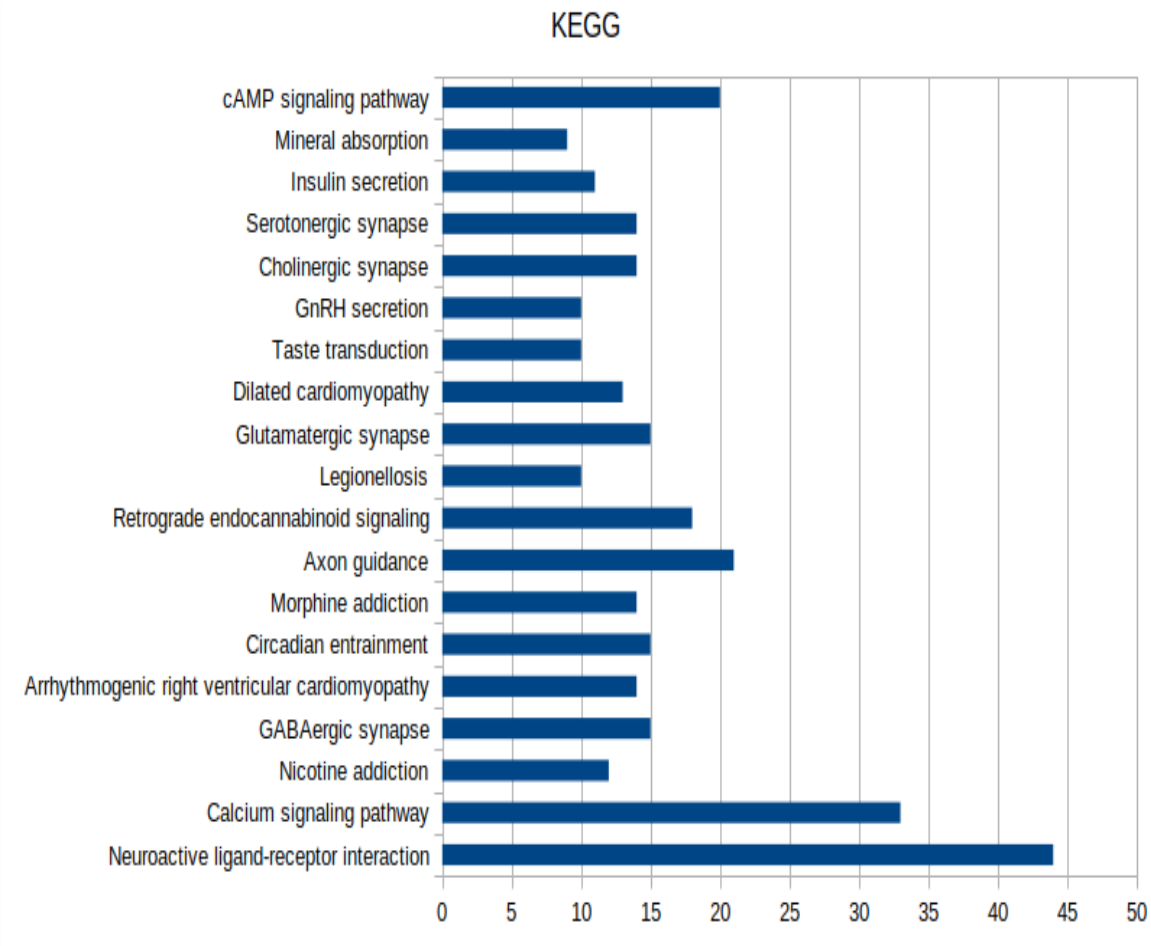

Figure 6.6 Statistically significant pathways affected from CdS NPS by KEGG based pathway enrichment analysis

Table 6.3: Top Ten enriched pathways (padj<0.001) of upregulated and downregulated DEGs associated with Cancer cells treated with CDS NPS

S. No	Pathways	DEGs	Adjusted p-value	Combined score	Genes involved
1	Calcium signaling pathway	Up	0.1583	12.28	RYR1; CHRM3; CHRM1; FLT1; PDE1C; CHRNA7; PTGER3; CACNA1B; HTR2C; ADCY2; CACNA1C; HTR4; ADCY8; CACNA1E; ADRA1A; RYR3; SLC8A1; GRM1; GRIN2A; GRM5; HTR7; ERBB4; PDGFD; PDGFC; CACNA1S; NOS1; NTRK2; NTRK3; TACR3; ATP2B2; MCOLN2; GDNF; SLC25A31
2	Mineral absorption	Up	0.9789	6.31	MT2A; MT1A; MT1F; HMOX1; MT1X; ATP2B2; SLC5A1; MT1B; SLC8A1
3	cAMP signaling pathway	Up	0.9999	1.16	GRIA1; GABBR2; GRIA2; CHRM1; HTR1F; PTGER3; ADCY2; ATP2B2; CACNA1C; HTR4; ADCY8; GRIN2B; TSHR; MAPK10; GRIN2A; FSHR; CNGB3; CACNA1S; VIP
4	Focal adhesion	Up	0.9999	0.91	SHC2; FLT1; VWF; LAMA2; LAMB4; THBS1; MYLPF; MAPK10; COL2A1; RELN; PDGFD; COL6A2; PDGFC;

					ITGA8; TNR; PAK3; PAK5
5	Apoptosis	Up	0.9999	0.51	MAPK10; NGFR; GADD45A; PTPN13; TNF; GADD45G
6	Folate biosynthesis	Down	0.4736	41.49	ALPP
7	Pentose phosphate pathway	Down	0.4736	33.94	H6PD
8	Nucleotide excision repair	Down	0.4736	17.82	GTF2H4
9	TNF signaling pathway	Down	0.4736	4.66	NFKBIA
10	Ras signaling pathway	Down	0.4983	1.27	EFNA1

KEGG pathway analysis elucidated that up and downregulated DEGs were enriched in a few pathways (refer: Table 6.1, 6.2, 6.3), including protein processing at the endoplasmic reticulum, folate, and thiamine metabolism, signalling in cancer and apoptotic pathways, transcriptional regulation in cancer, ion channels. In this study, FSHR, ADCY2, and ADCY8 were the genes involved in ovarian steroidogenesis, a process where ovary cells produce hormones for maintenance; previous studies also reported the involvement of these genes.[232] MAPK10 is the other major upregulated gene in the KEGG calcium signalling pathway seen in Figure 6. This gene is mainly involved in apoptosis, protein processing to ER, cell proliferation, and metastasis.[233] It can be said that the initial levels of MAPK10 resulted in cancer cell proliferation, but after the treatment with CdS NPs, MAPK10, in combination with pro-apoptotic microRNA, triggered the apoptotic pathway.[233], [234] Previous studies reported using MAPK10 as a potential target for cancer therapy including ovarian cancer.[235], [236]

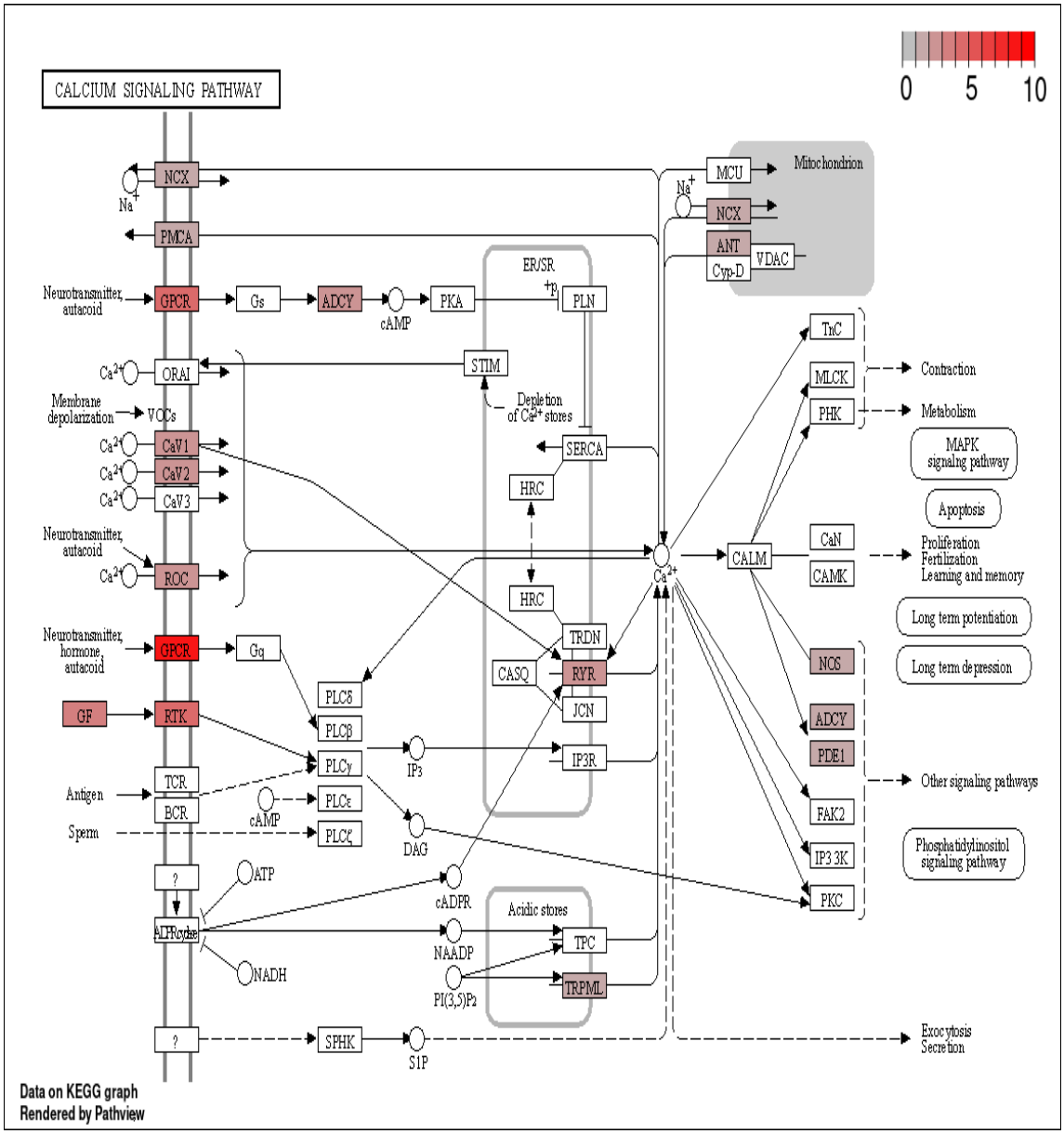

Figure 6.7 KEGG pathway analysis showing calcium signalling pathway inhibition reaches the highest percentage of expression.

Various biological essential pathways were downregulated, such as thiamine/folate metabolism (gene involved: ALPP). Thiamine metabolism is important for cell survival as thiamine plays an important role in PPP (pentose phosphate pathway), which is an alternative to glucose metabolism. In addition, also important in the metabolism of lipids, steroids, nucleic acid, etc., all these listed processes are very essential for the survival of cells.[237], [238] Another most

downregulated gene was NFKBIA which is generally responsible for the downregulation of many signaling pathways such as NOD-like receptor signaling pathway (NFKBIA + TXNIP), T cell and B- cell receptor signaling, IL-17 signaling, Toll-like receptor signaling, NF Kappa B signaling, TNF signaling to name some of them. EFNA1 gene downregulated a number of pathways responsible for cancer cell maintenance, such as JAK/STAT, MAP kinase, PI3-Akt, and microRNA in cancer signaling [230], which may be solely due to the introduction of a xeno-compound CdS NPS.

6.5. Conclusion

In conclusion, our investigation observed that the gene expression of CdS NPS-treated Cancer cells was significantly distinct from that of the untreated cell line. CdS NPS-treated cells demonstrated an inhibitory influence on the proliferation, migration, and signaling of cancer cells. By suppressing significant genes involved in a number of signaling pathways, notably MAP kinase, JAK/STAT, TNF signaling pathway PI3K-Akt, NOD-like receptor signaling, ECM-receptor interaction, microRNA pathways in cancer, Folate biosynthesis, etc., The investigation concluded by emphasizing that CdS NPS may have certain therapeutic effects on the Cancer cell line. However, more research will be required to confirm these results in various cell lines and in vivo environments in order to present new perspectives for cancer treatment advancements.