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Analysis of Pathways in Triple-Negative Breast Cancer Cells Treated with the Combination of Electrochemotherapy and Cisplatin

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Abstract: More than 2 million new cases and over 600,000 breast cancer deaths were reported in 2018 worldwide. Out of these, 15 to 20% are Triple Negative Breast Cancer (TNBC), which lack all the three most commonly administered receptors, namely ER, PR, and Her2 amplification. Hence, TNBC is difficult to treat; and it has the highest five-year recurrence rate among breast cancer types. Currently, TNBC patients are treated with platinum-based chemotherapeutics, such as cisplatin. With the aggressive and metastatic nature of TNBC cells, it demands immediate, alternate treatments. Electrochemotherapy is a proven drug delivery practice in molecular medicine. The combination of electrical pulses (EP)with Cisplatin (CsP)is studied using Label-free quantitative proteomics to better understand action pathways. Cisplatin alone and cisplatin combined with Electroporation (EP+CsP) on MDA-MB-231, human TNBC cells were used for this purpose. The results indicate that EP + CsP significantly upregulated Mitochondrial ribosomes and significantly downregulated ribosomes and ubiquitin-mediated proteolysis. A total of 12 proteins were found downregulated among both ribosomes and ubiquitin-mediated proteolysis. A total of 29 proteins were upregulated among Ribosomes. Mitochondrial ribosomes upregulation indicates the DNA damage was done by cisplatin, and proteasome inhibitors are proven to function as novel anticancer compounds.

Keywords: triple negative breast cancer; cisplatin; electroporation; upregulation; down regulation.

1. Introduction

Breast Cancer is the most commonly diagnosed cancer type among females, with the highest death rate [1]. Among all types and forms of Breast Cancer, Triple Negative Breast Cancer (TNBC) constitutes roughly 15 to 20% [2]. The majority of the TNBC cells fall under basal-like carcinoma [3] and lack all the three most commonly administered receptors, namely estrogen (ER), progesterone (PR), and human epidermal growth factor receptor 2 (HER2) amplification [4]. Chemotherapeutics commonly targets the receptors in order to inhibit cancer cell proliferation. The lack of all three receptors makes TNBC is the most difficult to treat. TNBC cells also have the highest five-year recurrence rate [5] among breast cancer types with the lowest 5-year survival rate.TNBC patients are commonly treated with platinum-based chemotherapeutics like cisplatin. The aggressive and metastatic nature of Triple-Negative Breast Cancer cells and the side effects of chemotherapeutics degrades the quality of life for TNBC patients. Hence TNBC demands immediate, alternate treatments.

Cisplatin [cis-DDP, cis-diamminedichloroplatinum(II)] is a platinum-based chemotherapy drug commonly used to treat various types and forms of cancer [6]. Cisplatin was initially discovered in 1845, but it was later identified that the platinum rods used as electrodes while electrolysis produced a platinum-based compound, which blocked cell division in Escherichia coli bacteria [7]. This compound gave rise to the discovery of cis-[PtCl2(NH3)2], which inhibited malignant tumor development in rats. This compound in 1978 was granted approval by the United States Food and Drug Administration. Cisplatin channels many pathways in both are upregulating them and downregulating them to inhibit TNBC cell proliferation [8]. Prior studies indicate the combination of Electroporation (EP) and Cisplatin (CsP) can induce controlled cell death by changing the metabolic activities in the Triple Negative Breast Cancer cells by employing the synergy of multiple metabolic pathways [9].

Electrochemotherapy is chemotherapy followed by electroporation [10, 11]. Electroporation is the process by which drugs are introduced into cells, followed by the application of electric pulses to the tumor area. Electric pulses are applied through a pair of metal electrodes in a limited tissue area where the tumor is present. The membranes of tumor cells are permeabilized, and injected drugs are absorbed into the cell readily. Electroporation facilitates drug transport through the cell membrane for those molecules that are normally non-permeant and hydrophilic. Two drugs have been identified to be effective when coupled with electroporation. Bleomycin, which is hydrophilic, could be potentiated 1000 times with electroporation [12, 13]. Cisplatin, another drug with limited transport through the cell membrane, showed an 80-fold increase in cytotoxicity with electroporation [14].

Electrochemotherapy (ECT) is an efficient modality to treat solid tumors. It applies to all types of histologies of cancer, such as melanoma, carcinoma, sarcoma. It has shown success in the clinics [15-19]. Typically bleomycin and cisplatin are used. When used intratumorally, cisplatin-based ECT offers a better-tolerated alternative to bleomycin-based ECT [19]. Recently, Kis et al. successfully performed bleomycin-based ECT for challenging eyelid-periocular Basal Cell Carcinoma and obtained a complete response in advanced primary and recurrent eyelid cases BCCs [20].

To understand the cellular mechanisms after electrical pulse application, conventional low throughput techniques were used to look just at a dozen proteins involved [21, 22]. However, given the complex signaling present in cancer cells, where thousands of proteins come together to form the whole-cell signaling network, no single cellular response could be accomplished by an individual protein. Therefore, a systemic investigation of the mechanism using a high-throughput technique [9, 23-27] is necessary to identify targets and off-targets for effective application of electrochemotherapy. Label-free quantitative proteomics provides a mechanism of action among cisplatin alone and cisplatin combined with Electroporation on MDA-MB-231, human TNBC cells [9].

2. Materials and Methods

The cells, drug, pulses used, and proteomics and statistical analyses were in [9]. In brief, MDA-MB-231, a human breast cancer cell line, originated from Caucasian breast adenocarcinoma, was used (ATCC®, USA) (Mittal, 2019). The MDA-MB-231cells were cultured at 37 °C, 70–80% humidity, and 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) (Gibco™, USA) supplemented with 1% Penicillin-Streptomycin and 10% FBS (Corning™, USA). For treatment, cells were detached using trypsin, were centrifuged at 1000 rpm for 5 min at 4 °C, and were resuspended in fresh media solution at 1×10^6 cells/mL.

Cisplatin (Sigma-Aldrich) at a concentration of 100 µM, was utilized. Eight, 1200V/cm, 100 µspulses were applied using a BTX-ECM830 electroporator (Genetronics Inc., USA). All experiments were performed in triplicates.

For proteomics study**,** LC-MS/MS data were collected using a reverse-phase HPLC-ESI-MS/MS system a combination of UltiMateTM 3000 RSLCnanoand a Q-Exactive (QE) High Field (HF) Hybrid Quadrupole OrbitrapTM MS (Thermo Fisher Scientific) and a Nano-spray FlexTM ion source (Thermo Fisher Scientific) and processed using MaxQuant (v1.6.1.0)71,72 against the Uniprot Homo sapiens fasta as in [9]. Statistics analyses were also done as in [9].

3. Results and Discussion

LC-MS/MS data using Q ExactiveOrbitrap HF hybrid MS coupled using UltiMateTM 3000RSLCnano HPLC system [9]. The tandem mass spectra were compared to the UniProt human protein database. Proteins identified in at least two of the three biological replicates at 1% FDR and at least 2 MS/MS (spectral) counts were considered for analyses. LFQ values ≠ 0 and MS/MS counts ≥ 2 were used. Fig. 1 shows the Venn diagram of the protein distribution. There were 2477proteins in CsPand 2457 in EP + CsP treatment, of which 2175 were commonly expressed in both samples. The number of uniquely identified proteins were 302 in CsP, and 282 in EP + CsP. The existence of unique proteins suggests a significant impact of EP on cellular pathways by increasing or decreasing many proteins. Compared to CsP, 547 proteins were upregulated, and 507 were downregulated in the EP + CsP treatment. Table 1 shows the top 20up and downregulated proteins and genes identified in this study.

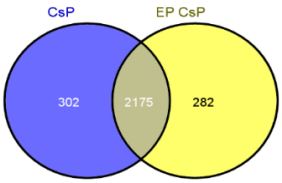

Figure 1. Venn diagram of protein distribution.

Table 1. Top 20 up-and down-regulated genes.

Protein		**Gene**	
Up	**Down**	**Up**	**Down**
B4DRR0	A0A024R5Q1	KRT6A	hCG_2003792
C0JYZ2	B3KM90	TTN	HCTP4
Q5QP19	A0A090MEW7	NFS1	HLA-C
H0YIV9	V9HW40	GATC	HEL-S-25
A0A024R8L7	A0A024RA49	ACOX1	ANLN
Q9BU61	Q658N3	NDUFAF3	DKFZp666G145
A0A1B0GTB8	Q5JSH3	CPT2	WDR44
B3KX82	O94973	SYVN1	AP2A2
B7Z705	P84157	CHID1	MXRA7
H7C1U8	C9JBI3	APOO	PSPH
B3KVJ8	A6NMQ3	CLCN7	ENSA
Q8IXM3	Q9NZL4	MRPL41	HSPBP1
A0A024R8D4	Q8WXV6	MRPS2	PLEC1
P42766	A0A024R0R4	RPL35	SAE1
Q9NUQ7	B4DWA2	UFSP2	CASP7
A0A087X1G7	P32456	TMEM97	GBP2
J3KT68	Q8IVM0	ECHDC1	CCDC50
Q9NTX5	B2R642	BCKDHA	MCAM
Q59EI3	J3KS94	NDUFB3	MBP
A0A024R413	A0A024R7G6	DDX18	EPS15L1

David 6.8 [28] was used to study the differentiated pathways, both up and down-regulated pathways, and Figures 2-5 were generated using the same.

3.1. Upregulated pathways.

3.1.1. Ribosomal and mitochondrial ribosomal proteins.

Both Mitochondrial ribosomal proteins(MRP) and ribosomal proteins (RP) were significantly upregulated in EP +CsP. Tables 2 and 3 give the list of RP and MRP identified from proteomics data. Here L refers to a large subunit, and S refers to a small subunit (Ribosomes are made up of two different subunits, which are needed for translation; where S does decoding of the genetic message and L catalyzes peptide bond formation. The essential features are largely conserved from yeast to humans) [29]. There are more large subunits identified in both mitochondrial ribosomes and ribosome proteins. This indicates that the formation of more peptide bond formation and protein release, indicating the effect of electrical pulses+cisplation in performing this compared to cisplatin alone.

Table 2. List of mitochondrial ribosome proteins identified from proteomics results.

Mitochondrial ribosomal proteins	
Large Subunit (L)	**Small subunit (S)**
MRPL1	MRPS10
MRPL11`	MRPS14
MRPL13	MRPS17
MRPL15	MRPS18A
MRPL19	MRPS2
MRPL37	MRPS22
MRPL38	MRPS23
MRPL39	MRPS27
MRPL4	MRPS36
MRPL41	MRPS5
MRPL43	
MRPL44	
MRPL46	
MRPL48	
MRPL49	
MRPL50	
MRPL53	

Table 3. List of ribosome proteins identified from proteomics results.

Ribosomal proteins	
Large Subunit (L)	**Small subunit (S)**
RPL13	RPS11
RPL14	RPS16
RPL17	RPS8
RPL18	RPS9
RPL18A	
RPL24	
RPL26	
RPL27	
RPL28	
RPL35	
RPL4	
RPL6	
RPL7	
RPL7A	
RPL8	

The large and small mitochondrial ribosomal proteins and ribosomal proteins are shown in Figure 2. The upregulated protein are highlighted by the red star sign in this Figure 2. Some

of the upregulated ribosomal proteins are mitochondrial ribosomal protein L1(MRPL1), mitochondrial ribosomal protein L13(MRPL13), ribosomal protein L13(RPL13), ribosomal protein L18a(RPL18A. The upregulation of ribosomal proteins validates the DNA damage done by cisplatin on TNBC cells [30].

Ribosomes are complex biological entities with numerous structural and accessory proteins [31]. This corroborates well with our proteomics results. The ribosomal proteins upregulated in our samples include: The Ribosomal proteins-large subunit (RPL) are: RPL 13, 14, 17, 18, 18A, 24, 26, 27, 28, 35, 4, 6, 7, 7A, 8, N1. The Ribosomal proteins-small subunit (RPL) are: RPS11, 16, 8, and 9.

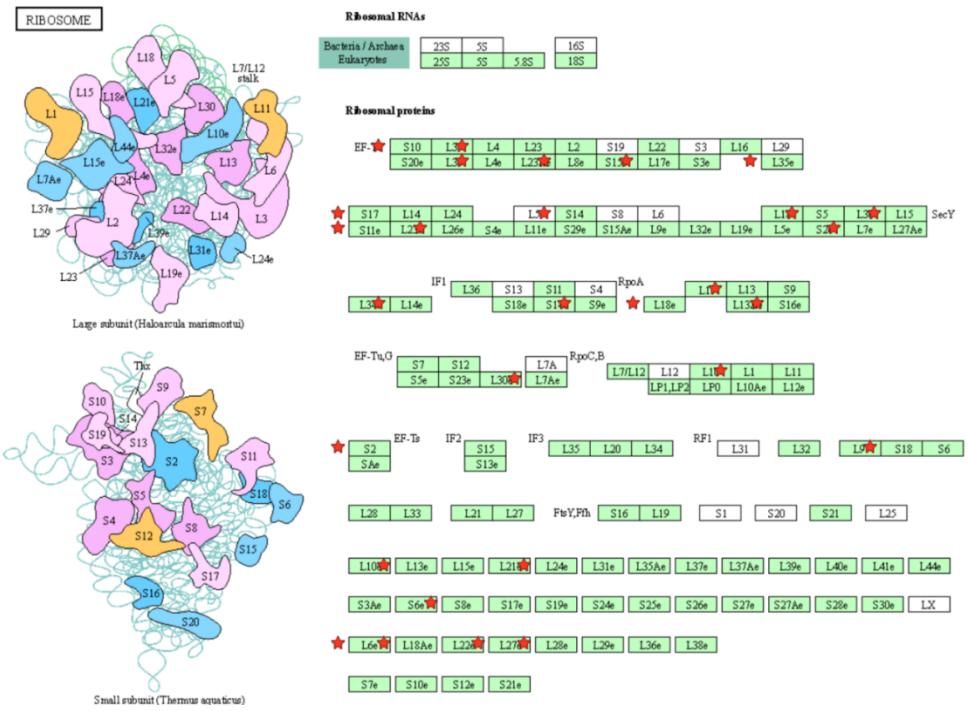

Figure 2. Ribosomal large and small subunits.

3.1.2. Pyruvate metabolism.

The multifaceted pyruvate metabolism [32] were also upregulated with EP + CsP, treatments. Figure 3 shows the pyruvate metabolism pathway. The highlighted regions in the metabolism shown in Figure 3 are Acetyl-CoA acetyltransferase 1(ACAT1), Aldehyde dehydrogenase 1 family member B1(ALDH1B1), Aldehyde dehydrogenase 2 families (mitochondrial)(ALDH2),Dihydrolipoamide S-acetyltransferase(DLAT), Fumarate hydratase(FH), Malate dehydrogenase 2(MDH2), Malic enzyme 2(ME2), Phosphoenolpyruvate carboxykinase 2, Mitochondrial(PCK2),Pyruvatecarboxylase(PC), Pyruvate dehydrogenase (lipoamide) alpha 1(PDHA1), and Pyruvate dehydrogenase (lipoamide) beta(PDHB). Lactate production in abidance is seen as a common trait in TNBC cell proliferation in aerobic and anaerobic respiration. This process is shunted by pyruvate production and metabolism, where pyruvate is sent to The Citric Acid Cycle (TCA cycle). The upregulation of Pyruvate metabolism shows that the Lactate production pathway is inhibited, which inhibits TNBC cell proliferation [33, 34].

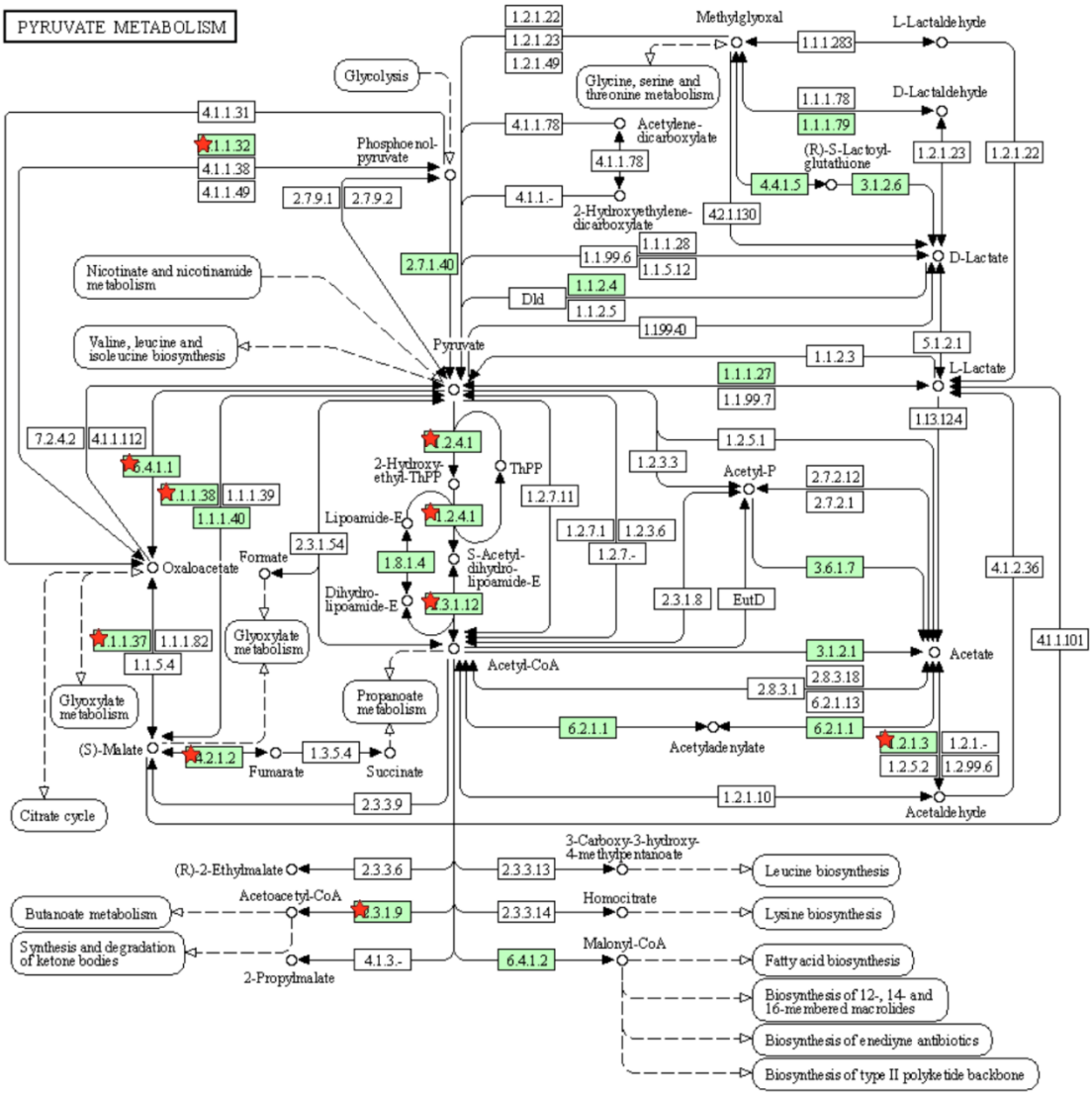

Figure 3. Pyruvate metabolism pathway.

3.2. Downregulated pathways.

Ubiquitin-mediated proteolysis and Proteoglycans in cancer and Ubiquitin-mediated proteolysis pathways were observed to be significantly downregulated in EP+CsP treatments (Figures 4 and 5).HECT and RLD domain containing E3 ubiquitin-protein ligase 4(HERC4), HECT, UBA and WWE domain containing 1, E3 ubiquitin-protein ligase(HUWE1), transcription elongation factor B subunit 1(TCEB1) are the genes which are highlighted in Ubiquitin-mediated Proteolysis shown in Figure 4. Table 4 gives the list of downregulated UB proteins observed in our study.

The genes actively involved in the Proteoglycans in cancer pathways, which are downregulated, are IQ motif containing GTPase activating protein 1(IQGAP1), KRAS proto-oncogene, GTPase(KRAS), actin gamma 1(ACTG1), and mitogen-activated protein kinase 1(MAPK1), as highlighted in Figure 5. Proteoglycans in cancer which aid the TNBC cells in adhesion and tumor progress are downregulated. Similarly, Ubiquitin-mediated Proteolysis pathways are downregulated; such proteins are responsible for the breaking of protein molecules. Inhibiting the Ubiquitin-proteosome pathway will result in blocking of cell cycle development [35]. It also suggests that the proteasome inhibitors can be utilized for

multifarious tumor cells. Proteoglycans influence cancer cells' behavior and their microenvironment during the progression of solid tumors [36]. Also, suppression of ubiquitin-mediated proteolysis and promotion of protein synthesis is reported by [37]. The downregulation of these two in our study correlates well with this.

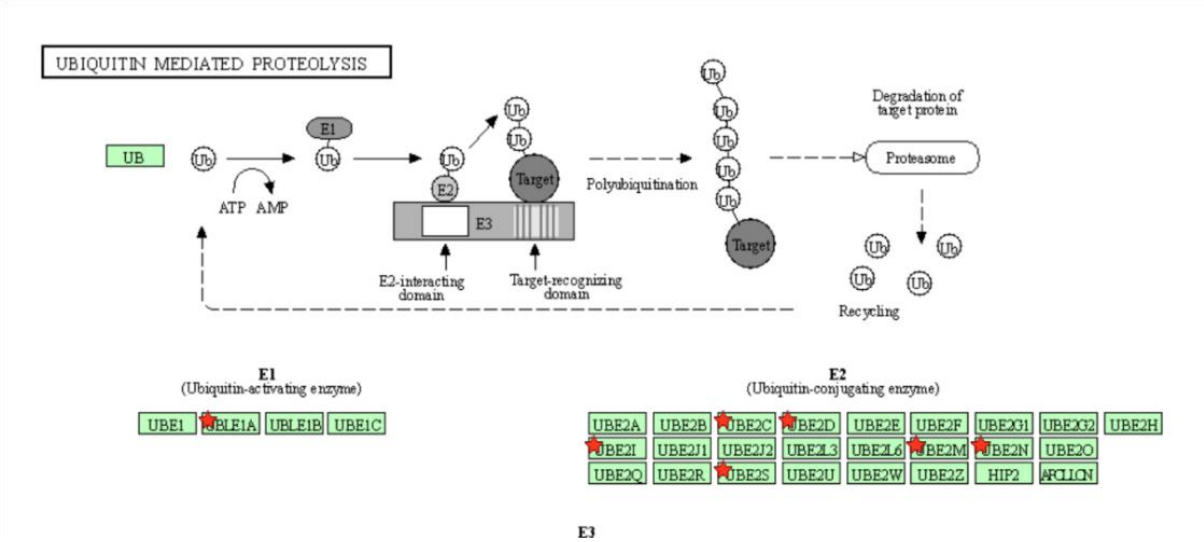

Figure 4. Ubiquitin mediated proteolysis.

Table 4. List of ubiquitin proteins identified from proteomics results.

Ubiquitin proteins downregulated
UBA2
UBAP2L
UBE2C
UBE2D3
UBE2E2
UBE2I
UBE2M
UBE2S
UBQLN1

The other downregulated genes include CASP7, MAPK1, MAPK14, ENO1, ENO2, and LDHB. CASP7 is a protein-coding gene and is directly involved in apoptosis modulation and signaling. This gene's downregulation is associated with more cell death, which is the preferred action in this study. MAPK genes are associated with mediating with intracellular signaling. They transduce signals from growth factors. Again, downregulation of these genes is preferred. ENO1 overexpression is associated with several tumors, including breast cancer and lung cancer.

3.3. Gene upregulation and downregulation in the biological process.

Analysis of gene upregulation and gene downregulation present in the Biological Processes were studied and categorized in Figures 6 and 7, using Genecodis [38]. Figure 6 shows the top ten upregulation based on the number of genes. The highest number of genes were found in the maturation of LSU-rRNA from tricistronicrRNAtranscript and fatty acid beta-oxidation with more than 4 genes present in each of the processes.

Figure 7 shows the top ten downregulation based on the number of genes. In this case, translation and neutrophil degranulation were seen to be containing the maximum genes in the downregulation processes. The translation process has more than 50 genes affected, and neutrophil degranulation has more than 40 genes downregulated.

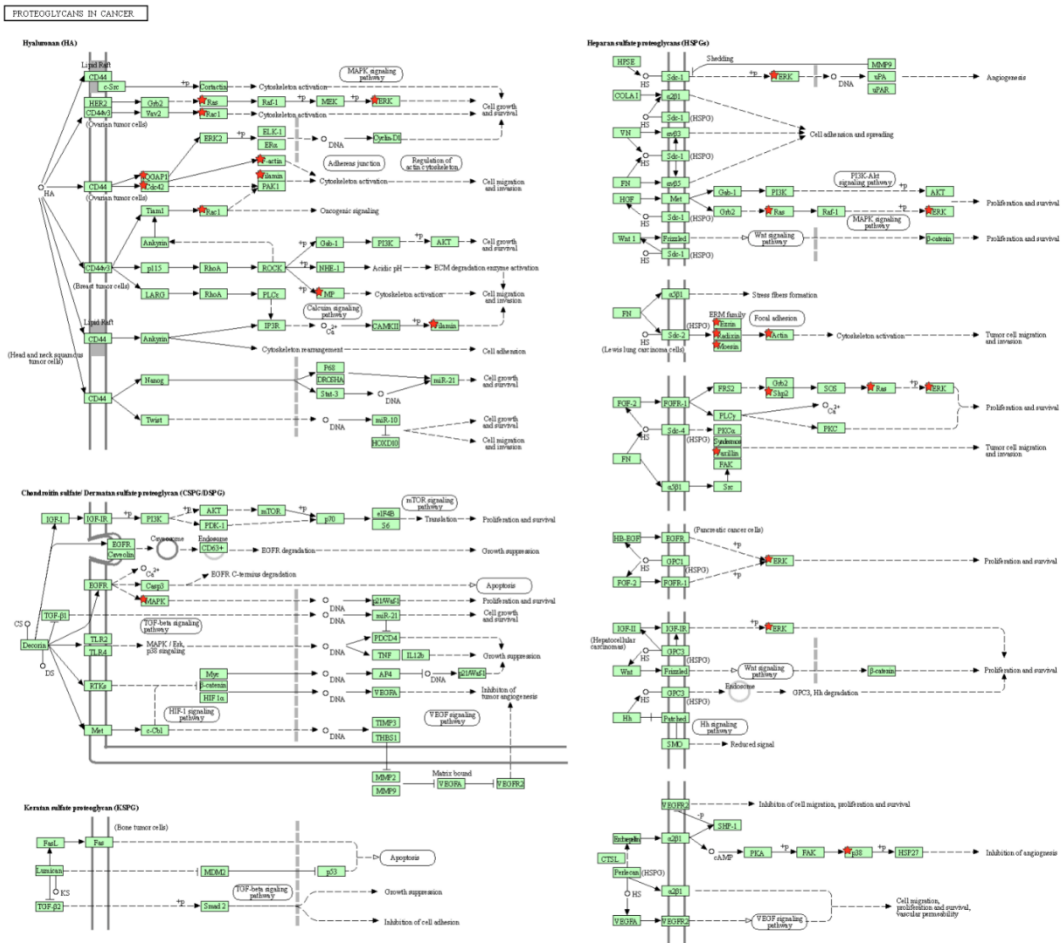

Figure 5. Proteoglycans in cancer.

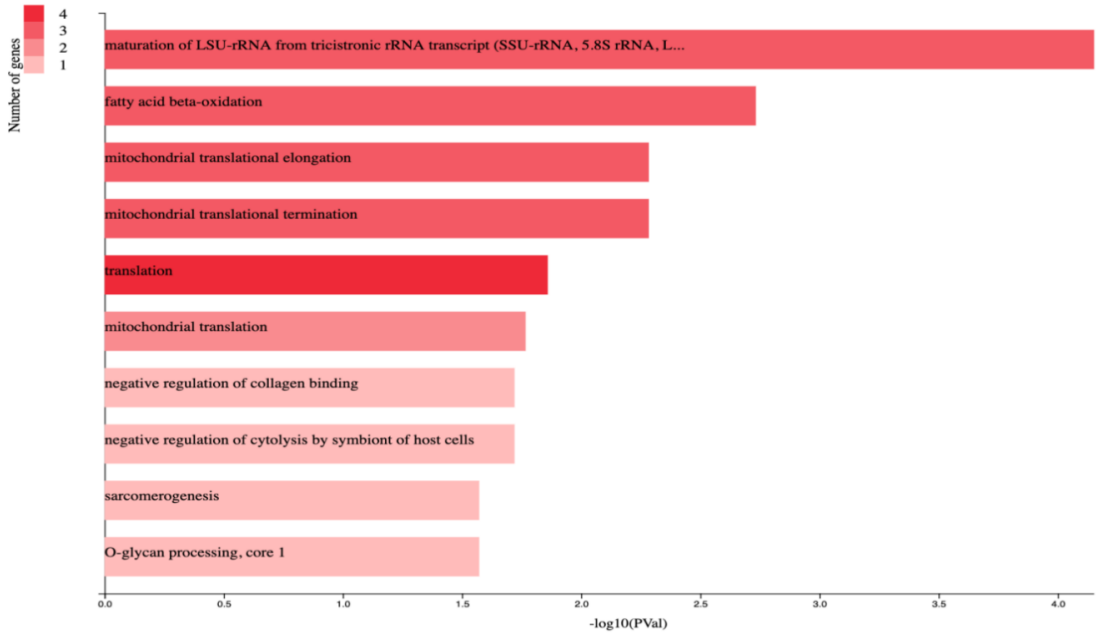

Figure 6. Top 10 upregulation based on several genes.

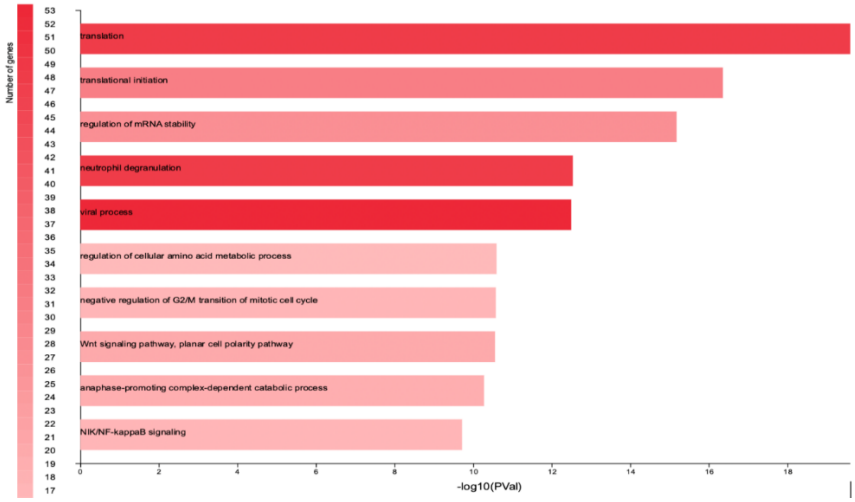

Figure 7. Top 10 downregulation based on several genes.

The mechanism of action of cisplatin involves binding to the DNA strands and influence key cellular functions [39]. Initially being a neutral molecule, it gets positively charged, with the presence of water, by the displacement of a chloride ligand and becomes an active monoaquated complex 1, as shown in Figure 8 [39]. Being positively charged, next it is directed to negatively charged nucleic acids directs it to negatively charged nucleic acids. The active species coordinates to the N7 of purine bases and forms stable adducts on DNA. Although DNA is considered the major target of cisplatin, RNA and proteins are also susceptible to platinum-adduct formation. Also, cisplatin cytotoxicity modulates compounds that target the ribosome and inhibit protein synthesis. The up-regulation of ribosomal and mitochondrial ribosomal proteins in our study correlates well with this. This is also corroborated by the viability studies [9]. The EP+CsP samples show lower viability of less than 5%, compared to the higher viability of 80%, with drug alone, indicating the strong impact of electrical pulses when combined with cisplatin. This combination therapy could be used to alleviate the drug resistance [40-42] issues seen eventually in the patients who respond initially.

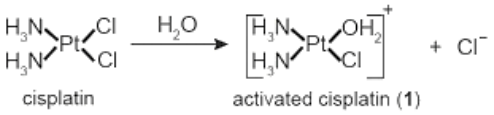

Figure 8. Cisplatin combining with water releases chloride ions.

4. Conclusions

TNBC tumor development comprises complex signaling in metabolic pathways to aid proliferation, where multiple pathways with the help of proteins come together to form the cell signaling network. Analysis of upregulation and downregulation of pathways due to the combination of EP + CsP provides us insight into metabolic pathways. These pathways are being inhibited by downregulating ubiquitin-mediated proteolysis and Proteoglycans in cancer, suggesting the inhibition of TNBC cell proliferation and upregulation of mitochondrial ribosomes and pyruvate metabolism, which aids inhibition of TNBC cell proliferation. Mitochondrial ribosomes upregulation indicates the DNA damage done by cisplatin and proteasome inhibitors are proven to function as novel anticancer compounds. A total of 12 proteins were found downregulated among both ribosomes and ubiquitin-mediated proteolysis. A total of 29 proteins were upregulated among ribosomes.

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Conflicts of Interest

The authors declare no conflict of interest.

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