# Appendix

## List of Publications

1. **Manoj Sharma**, Upalabdha Dey, Anindhya Sundar Das, Kaushika Olymon, Aditya Kumar, and Rupak Mukhopadhyay. "Anti-tumor potential of high salt in breast Cancer cell lines." Molecular Biology Reports 51, no. 1 (2024): 1002.

2. Basumatary Mandira, Amit Talukdar, **Manoj Sharma**, Anupam Dutta, Rupak Mukhopadhyay, and Robin Doley. "Exploring the anticancer potential of Cytotoxin 10 from Naja kaouthia venom: Mechanistic insights from breast and lung cancer cell lines." Chemico-Biological Interactions (2024): 111254.

3. Majumder Munmi, **Manoj Sharma**, Siddhartha Maiti, and Rupak Mukhopadhyay. "Edible tuber Amorphophallus paeoniifolius (dennst.) extract induces apoptosis and suppresses migration of breast cancer cells." Nutrition and Cancer 73, no. 11-12 (2021): 2477-2490.

4. Das Anindhya Sundar, Anandita Basu, Ravi Kumar, Pallab Kumar Borah, Subhojit Bakshi, **Manoj Sharma**, Raj Kumar Duary, Partho Sarothi Ray, and Rupak Mukhopadhyay. "Post-transcriptional regulation of C-C motif chemokine ligand 2 expression by ribosomal protein L22 during LPS-mediated inflammation." The FEBS journal 287, no. 17 (2020): 3794-3813.

5. Deka Archana, **Manoj Sharma**, Rupak Mukhopadhyay, Arpita Devi, and Robin Doley. "Naja kaouthia venom protein, Nk-CRISP, upregulates inflammatory gene expression in human macrophages." International Journal of Biological Macromolecules 160 (2020): 602-611.

6. Basu Anandita, Anindhya Sundar Das, **Manoj Sharma**, Manash Pratim Pathak, Pronobesh Chattopadhyay, Kaushik Biswas, and Rupak Mukhopadhyay. "STAT3 and NF-κB are common targets for kaempferol-mediated attenuation of COX-2 expression in IL-6-induced macrophages and carrageenan-induced mouse paw edema." Biochemistry and biophysics reports 12 (2017): 54-61.

7. Deka Archana, Maitreyee Sharma, **Manoj Sharma**, Rupak Mukhopadhyay, and Robin Doley. "Purification and partial characterization of an anticoagulant PLA2 from the venom of Indian Daboia russelii that induces inflammation through upregulation of proinflammatory mediators." Journal of Biochemical and Molecular Toxicology 31, no. 10 (2017): e21945.

## Oral presentation and Conferences

1. Manoj Sharma. Role of high dietary salt in *inflammation-induced anti-tumorigenicity.* National Seminar on *"Biology is Fascinating".* March. 2022. *Organized by Department of Molecular Biology and Biotechnology, Tezpur University India (**Oral Presentation)***

2. Manoj Sharma, Anindhya Sundar Das, Anindita Basu, Munmi Majumder and Rupak Mukhopadhyay. *High dietary salt induces inflammation-induced anti-tumorigenicity. International Symposium on "Emerging Trends and Challenges in Cancer Chemoprevention, Diagnosis and Therapeutics (CancerSymp-2020)" February 17-18, 2020. Organized by Department of Molecular Biology and Biotechnology, Tezpur University India **(Poster Presentation)***

Molecular Biology Reports (2024) 51:1002
https://doi.org/10.1007/s11033-024-09925-4

ORIGINAL ARTICLE

# Anti-tumor potential of high salt in breast Cancer cell lines

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Received: 29 June 2024 / Accepted: 9 September 2024


## Abstract

**Background** Recent $^{23}$Na-MRI reports show higher salt deposition in malignant breast tissue than in surrounding normal tissue. The effect of high salt on cancer progression remains controversial. Here, we investigated the direct effect of high salt on breast cancer progression in vitro.

**Methods** Here, the impact of high salt on apoptosis, proliferation, cell cycle, adhesion, and migration of MDA-MB-231 and MCF-7 cells was studied using MTT, scratch, and clonogenic assays, as well as RT-PCR and flow cytometry. Gene expression was analyzed using Real-Time PCR and western blotting. The effect of high salt on global transcriptomics changes in MDA MB-231 cells was studied using RNA-sequencing analysis.

**Results** Flow cytometry with Annexin V and CFSE revealed that high salt-induced dose-dependent apoptosis and inhibited proliferation. High salt-induced cell cycle arrest at the G1/S phase of the cell cycle. p-MDM2 is known to suppress p53, which plays a crucial role in regulating apoptosis and cell cycle arrest under cellular stress conditions. High salt treatment led to decreased p-MDM2 and increased p53 expression, suggesting that high salt induces apoptosis through p53 stabilization. decreased p-MDM2 and increased p53 expression. High salt also reduced migration and adhesion of cells in a dose-dependent manner suggesting its inhibitory effect on metastatic properties as evident from wound healing assay. RNA sequencing analysis revealed overexpression of tumor suppressor genes and genes associated with anti-tumor activity (PCDHGA11, EIF3CL, RAVER1, TNFSF15, RANBP3L) and under-expression of genes involved in cancer-promoting activity (MT1X, CLDN14, CSF-2).

**Conclusion** Our results unequivocally demonstrate the anti-tumor efficacy of high salt against breast cancer cells, suggesting its potential as a therapeutic strategy in cancer treatment.

**Keywords** High salt · Breast cancer · MDA MB-231 · MCF-7 · Apoptosis · Cell proliferation · Cell cycle · Migration · Wound healing · Adhesion · RNA-sequencing

**Abbreviations**

| | |
|---|---|
| GAG's | Glycosaminoglycans |
| MDSCs | Myeloid-derived suppressor cells |
| ROS | Reactive oxygen species |
| DEGs | Differentially expressed genes |
| ECM | Extracellular matrix |

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Published online: 21 September 2024

## Introduction

Breast cancer is one of the most prevalent types of cancer globally, accounting for 12.5% of annual cancer cases worldwide. In 2020, 2.3 million women were diagnosed with breast cancer leading to 685,000 deaths globally [1]. Dietary salt (NaCl) serves as a vital nutrient, crucial for preserving cellular homeostasis, regulating fluid and electrolyte balance, and managing blood pressure [2]. It plays a pivotal role in maintaining extracellular fluid volume, exerting osmotic effects, facilitating muscle movement, transporting nutrients and substrates across the plasma membrane, and maintaining nerve cell excitability [3]. However, high-salt diet is known for their adverse impact on human health, contributing to the exacerbation of various conditions like hypertension, cardiovascular diseases, and autoimmune disorders [4]. The relation between high salt intake and cancer has not been extensively studied, and there is only a limited amount of literature on the association between salt consumption and cancer progression. $^{23}$Na-MRI, which enables noninvasive measurement of tissue sodium concentrations, revealed a significant difference in sodium ion concentration between normal and malignant tissues [5, 6]. Although the factors leading to diet-independent accumulation of local sodium remain unclear, it might be a result of an expanded interstitial space caused by alterations in cellular organization or due to impaired metabolic changes affecting sodium exchange across the cell membrane [7]. In contrast, a separate study proposed that tissue sodium accumulation might be, to some extent, influenced by the binding of positively charged ions to negatively charged glycosaminoglycans (GAG's) [8]. Such differences in sodium concentration might have significant functional implications in tumor microenvironment and overall outcome of cancer treatment and progression. High salt is known to have a potential effect on the polarization of immune cells in tumor microenvironment towards a pro-inflammatory state [9]. It promotes the generation of inflammatory IL-17-producing CD4 + T cells (Th17) in both mice and humans [10]. On the other hand, high salt hinders the immunosuppressive functions of regulatory T cells which plays an important role in inhibiting anti-cancer immune responses from immune cells in the tumor microenvironment [11]. The cells within the tumor microenvironment typically exist in an immunosuppressed state [12]. The pro-inflammatory state induced in immune cells under the influence of high salt could potentially enhance their transition from the immunosuppressed state to their immune-responsive state, thereby facilitating their effectiveness in combating the tumor.

The link between high salt intake and cancer is a topic of ongoing debate, with only a few studies suggesting a potential pro-tumorigenic effect of high salt. For example, dietary salt increases the risk of gastric cancer, contributing to both the incidence and mortality of the disease by damaging the gastric mucosal lining [13]. Other research indicated that a high salt diet could stimulate the growth of breast cancer and promote lung metastasis by elevating Th17 cell levels. This could potentially drive tumor progression through the secretion of IL-17 F and the activation of the MAPK signaling pathway in breast cancer cells [14]. In contrast, high salt-containing diet significantly inhibited tumor growth, primarily through the depletion and functional modulation of myeloid-derived suppressor cells (MDSCs), highlighting its anti-cancer efficacy [15]. High salt also reduces cytokine production, necessary for the expansion of MDSCs, and the accumulation of these cells in blood, spleen, and tumors [16]. As a result, the phenotypes of monocytic and granulocytic MDSCs shift towards anti-tumor macrophages and pro-inflammatory state respectively, thereby reactivating the anti-tumor actions of T cells. A high salt diet in mice also showed increased Bifidobacterium abundance and heightened gut permeability, leading to intratumor localization of Bifidobacterium, enhancing NK cell functions, and facilitating tumor regression [17]. This suggests potential implications of salts in cancer immunotherapy. However, the overall impact of high-salt diets on breast cancer, is not fully understood, emphasizing the need for further research to elucidate their influence on breast cancer cells and the tumor microenvironment. Our study demonstrated that high salt treatment increased apoptosis arrested the cell cycle in the G1/S phase, and inhibited proliferation, migration, and adhesion of both triple-negative MDA MB-231 and ER + MCF-7 breast cancer cell lines. Transcriptomic analysis of MDA MB-231 cells also revealed enhanced expression of genes involved in tumor suppression; these functionally important genes could help us understand and investigate the therapeutic role of these genes under the influence of high salt.

## Materials and methods

### Cell lines and reagents

The cell lines MDA-MB-231 and MCF-7 were purchased from NCCS Pune, India. Dulbecco's Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from Life Technologies, USA. MTT (3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide), Collagen I and IV, ECM gel, were purchased from Sigma Aldrich and Mitomycin-C was purchased from HiMedia, India. Antibodies used in this study were procured from Cell Signaling Technology, USA. Cell Trace CFSE Cell Proliferation and Annexin V Ready Flow

Conjugates kits were purchased from Thermo Fischer Scientific, USA.

## Cytotoxicity assay

10,000 cells were plated in each well of a 96-well plate and incubated overnight. After 24 h, cells were treated with different concentrations of NaCl or osmo-active agents like urea and mannitol for 24 h. At the end of the incubation period, cells were treated with MTT and incubated for 3 h. The media was removed carefully and MTT dissolving solution was added and absorbance was taken at 590 nm wavelength using a UV-visible spectrophotometer (Multiscan Go, Thermo Scientific).

## Apoptosis assay using flow cytometer

MDA-MB-231 and MCF-7 cells ($1 \times 10^5$ /well) were seeded in 12 well plates and incubated overnight. Adhered cells were treated with different concentrations of high salt for 24 h. After incubation, both floating as well as adherent cells from each well were collected in tubes and washed with PBS. The cell pellets were resuspended in 500 µl of binding buffer and Alexa fluor 488 conjugated Annexin V was added according to the manufacturer's protocol (Thermo Fischer Scientific, USA). The samples were then allowed to incubate in the dark for 15 min and then analyzed with CellQuest software using a FACSLyric flow cytometer (BD Biosciences, USA).

## Cell cycle analysis

MDA-MB-231 and MCF-7 cells were cultured overnight in complete media followed by a 24-hour incubation in serum-starved media. Cells were treated with high salt for 48 h in complete media. At the end of incubation, the cells were pelleted, washed with PBS, fixed in 70% ethanol overnight at − 20 °C and stained with a solution containing 100 µg/ml propidium iodide (PI) and 100 µg/ml RNAse-A. Cell cycle phases were estimated as a percentage of a total of 10,000 events. The DNA content was measured and the data acquired was analyzed using CellQuest software using FACSLyric flow cytometer (BD Biosciences, USA).

## Cell proliferation assay

CellTrace™ CFSE Cell Proliferation Kit (Thermo Fischer Scientific, USA) was used to measure cell proliferation in MDA MB-231 and MCF-7 cell lines. Cells were cultured in complete growth media till 80–90% confluency. Cells were then trypsinized, centrifuged at 300× *g*, washed, and resuspended in serum-free DMEM media. Cells were transferred to CFSE buffer to prepare a cell suspension of $1 \times 10^6$ cells/ml. The mixture was then incubated in a water bath at 37 °C for 15 min to bind intracellular proteins, primarily amine groups of lysine residues. Following the wash with serum-free media, cells were resuspended in complete growth media at 37 °C and incubated for 1 h. After washing and resuspending, $5 \times 10^5$ cells per well were seeded into a 100 mm dish and were allowed to proliferate for 7 days. The fluorescence of the CFSE-labelled cells was quantified using a FACSLyric flow cytometer (BD Biosciences, USA).

## Measurement of reactive oxygen species (ROS) level

ROS level was quantified using the chloromethyl derivative of 2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) (Thermo Fisher Scientific, USA), as per manufacturer's protocol. Briefly, MDA-MB-231 and MCF-7 cells were treated for 24 h with high salt in Corning® 96 Well Black Polystyrene Microplate. The treated cells were washed with PBS, resuspended in 10 µM CM-H2DCFDA, in PBS, and incubated at 37 °C, for 30–40 min. Subsequently, the stained cells were maintained in a fresh complete medium and incubated at 37 °C for 20 min followed by analysis in a multimode microplate reader (Varioskan LUX, Thermo Fisher Scientific, USA) at 495/520nm.

## Wound healing assay

MDA MB-231 and MCF-7 cells ($1 \times 10^6$ cells/ well) were seeded in 6 well plates to adhere to the surface followed by serum starvation for 12 h. One hour before the treatment with high salt, cells were treated with 10 µg/ml mitomycin-c to block the proliferation of cancer cells. A straight-line scratch was created with the help of a 10 µl sterile tip and cells were washed with 1x PBS to remove all non-adherent cells. The wound was imaged at 0, 24, and 48 h using a microscope (Model: Olympus IX83).

## Adhesion assay

Cells ($2 \times 10^5$ /ml) were pre-treated with different concentrations of NaCl in serum-free media for 24 h. Cells were then plated in 96-well plates pre-coated with collagen I and IV, and allowed to adhere for 60 min. The media was gently removed, and wells were washed with 1X PBS to remove all non-adherent cells. Cells were then incubated in a complete medium and the percentage of adhered cells was quantified using an MTT assay as described earlier.

### RT-PCR analysis

Total RNA was isolated using TRIzol™ Reagent (Invitrogen, USA) following the manufacturer's protocol. 1 µg of total RNA was converted into cDNA using Verso cDNA Synthesis Kit, (Thermo Fisher Scientific, USA). RT-PCR was performed using iTaq Universal SYBR Green Supermix, (Bio-Rad, USA). The primer sequences of the genes are provided in Supplementary Table 1.

### Western blot analysis

Cells were seeded in a 60 mm dish and incubated overnight before treating with the indicated concentrations of NaCl for 24 h. Proteins were extracted from MDA MB-231 and MCF-7 cells with ice-cold RIPA buffer (Thermo Scientific, USA) containing protease and phosphatase inhibitor cocktail (Thermo Scientific, USA). Equal concentrations of proteins from different experimental samples were run in SDS-PAGE and transferred to PVDF membrane using a semi-dry electrophoresis transfer unit (GE Healthcare, UK). The membranes were probed with the corresponding primary antibodies (1:1000 dilutions) overnight at 4° C followed by secondary antibodies for 1 h at room temperature. The blots were then incubated with a chemiluminescence substrate (Bio-Rad, USA) and bands were visualized using the Chemidoc XRS + system (Bio-Rad, USA). Quantification of the bands was done using ImageJ software.

### RNA sequencing analysis

Total RNA was extracted from MDA MB-231 control and treated cells (50 mM NaCl). Library preparation was performed using NEB Next®Ultra™ II RNA Library Prep Kit for Illumina® following the manufacturer's instructions. Further quality and quantity were assessed using the Agilent 4150 Tape Station system and Qubit 4 Fluorometer. High-quality total RNA-Seq libraries were sequenced ($2 \times 150$ bp) using the Illumina NovaSeq 6000 V1.5 platform. Quality control and pre-processing of raw reads obtained from sequencing in FASTQ format were performed using the open-source package fastp. Processed reads were aligned to human genome version 38 using Hisat2 tool to generate BAM files. Read counts were generated using the Feature Counts tool. Differential expression was calculated using the Bioconductor package edgeR. Absolute Log2foldchange of 1.5, and p-value less than 0.05 was used to define the differentially expressed genes (DEGs). EnhancedVolcano R package was used to generate the volcano plot. 'pheatmap' R package was used to plot the heatmap of the top 50 DEG sorted by p-values. Further, the DEG list was used for gene ontology (GO) and KEGG pathway over-representation analysis (ORA). GO over-representation results were obtained by using the enrichGO function from the clusterProfiler R package. For the KEGG pathway, enrichment analysis was performed with enrichKEGG. Other plots were generated using the ggplot2 R package.

### Statistical analysis

Statistical analysis was performed using Graph Pad Prism 9.0 and data were expressed as Mean ± Standard error. Results were analyzed either by student's t-test, one-way or two-way analysis of variance (ANOVA). The significance was calculated as required by the experimental system.

## Results

### High salt primes cancer cells towards apoptosis

To assess the effect of high salt on breast cancer cells, its cytotoxic potential was determined. Notably, high salt treatment exhibited a dose-dependent increase in cytotoxicity in MDA-MB-231 and MCF7 cell lines. Treatment with 100 mM NaCl for 24 h resulted in 32% and 41% cell death in MDA-MB-231 and MCF7 cells, respectively (Fig. 1A). Flow cytometry analysis using Annexin V and PI, showed a dose-dependent increase in apoptosis in both cell lines (Fig. 1B). Quantitative analysis of the flow cytometry data is represented in Fig. 1C. To understand the effect of high salt on the expression levels of the key apoptosis-regulating proteins, western blots were performed (Fig. 1D). High salt resulted in an increased expression of the apoptotic protein Bax while suppressing the anti-apoptotic protein Bcl-2 in a concentration-dependent manner in both cell lines. This orchestrated effect led to an elevation in the Bax/Bcl-2 ratio, a crucial marker for apoptotic cells. Treatment with high salt also led to upregulation of Caspase-7, indicating the initiation of apoptosis in the cells. An increase in ROS level is known to have a detrimental effect on cancer progression by inducing apoptosis [18]. High salt treatment showed a steady increase in ROS levels in both cells, suggesting a potential role in inducing apoptosis (Supplementary Fig. 1).

### Effect of high salt on cell cycle and cell proliferation

CFSE dye dilution method was used to study the effect of high salt on the proliferation of breast cancer cells.

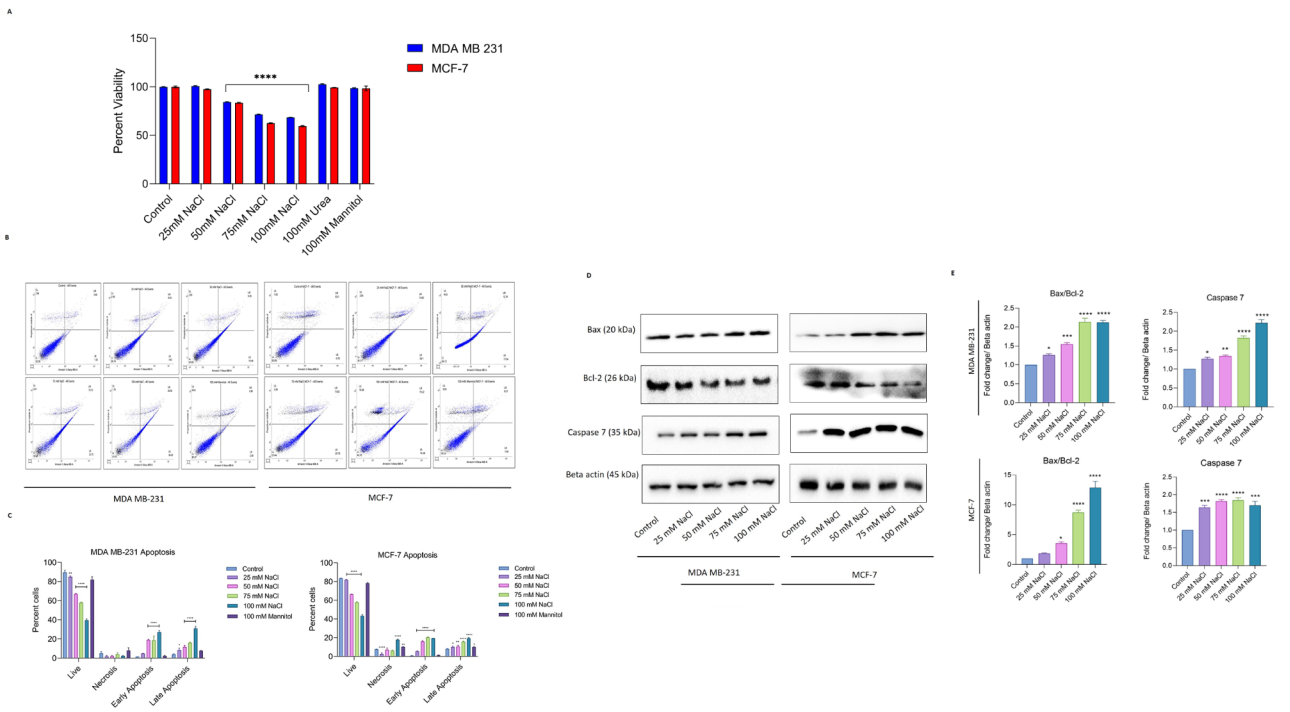


**Fig. 1** High salt induces apoptosis in breast cancer cell lines. **A**. Cell viability assay of MDA MB -231 and MCF-7 cell lines using MTT assay at 24 h. **B**. Flow cytometric analysis of high salt treated MDA MB-231 and MCF-7 cells using Annexin V/PI. **C**. Quantitative analysis of the percent live and dead cells. **D**. Western blot analysis of apoptosis markers in MDA MB-231 and MCF-7 cell lines. **E**. Densitometric analysis of blots using ImageJ software, NIH. (Statistical analysis by two-way ANOVA test. *$p < 0.05$, **$p < 0.005$, ****$p < 0.0005$)

CFSE, a fluorescent dye, is evenly distributed throughout the cytoplasm in a labeled cell. However, with each subsequent cell division, the dye is equally distributed in daughter cells, resulting in a decrease in fluorescence intensity. High salt treatment for 7 days suppressed the proliferation of both MDA MB-231 and MCF-7 cells (Fig. 2A-B). The quantitative analysis of the CFSE cell proliferation assay is represented in Fig. 2C-D. The effect was significant from the salt concentration of 50 mM onwards. However, treatment with 100 mM Mannitol did not affect the proliferation in both cells. The effect of high salt on cell proliferation was further confirmed using a clonogenic assay with varying salt concentrations. Our data showed inhibition of colony number and areas in both MDA MB-231 and MCF-7 cells, particularly at salt concentrations of 50 mM and beyond (Fig. 2E-F). Treatment with 100 mM mannitol showed no effect on the clonogenic properties of cancer cells suggesting the inhibition of clonogenic activity by salt was not due change in osmolarity. This suggests that increased salt concentrations, especially at and beyond 50 mM, may play a role in impeding clonogenic survival.

## High salt arrests the cell cycle of breast cancer cells in the G1/S phase

To delve deeper into the intracellular mechanisms underlying the inhibitory impact of high salt on breast cancer cell proliferation, we investigated the cell cycle distribution in MDA-MB-231 and MCF7 cells using propidium iodide (PI) based flow cytometric analysis. In comparison to the control, high salt-treated cells exhibited distinct cell cycle arrest at the G1/S phase. (Fig. 3A-B). Interestingly, high salt treatment also reduced the expression of Cyclin D1 (Fig. 3C-D). p-MDM2 is known to suppress p53 which plays a crucial role in regulating apoptosis and cell cycle under cellular stress. High salt treatment showed a decrease in p-MDM2 and an increase in p53 expression suggesting apoptosis promoting role of high salt through the stabilization of p53 (Fig. 3C-D).

## High salt suppresses migration and adhesion in breast cancer cell lines

Cell migration plays a crucial step in initiating cancer metastasis, enabling it to spread to various organs. To investigate the effect of high salt treatment on breast cancer cell migration, both MDA MB-231 and MCF-7 cells were treated with various concentrations of salt for

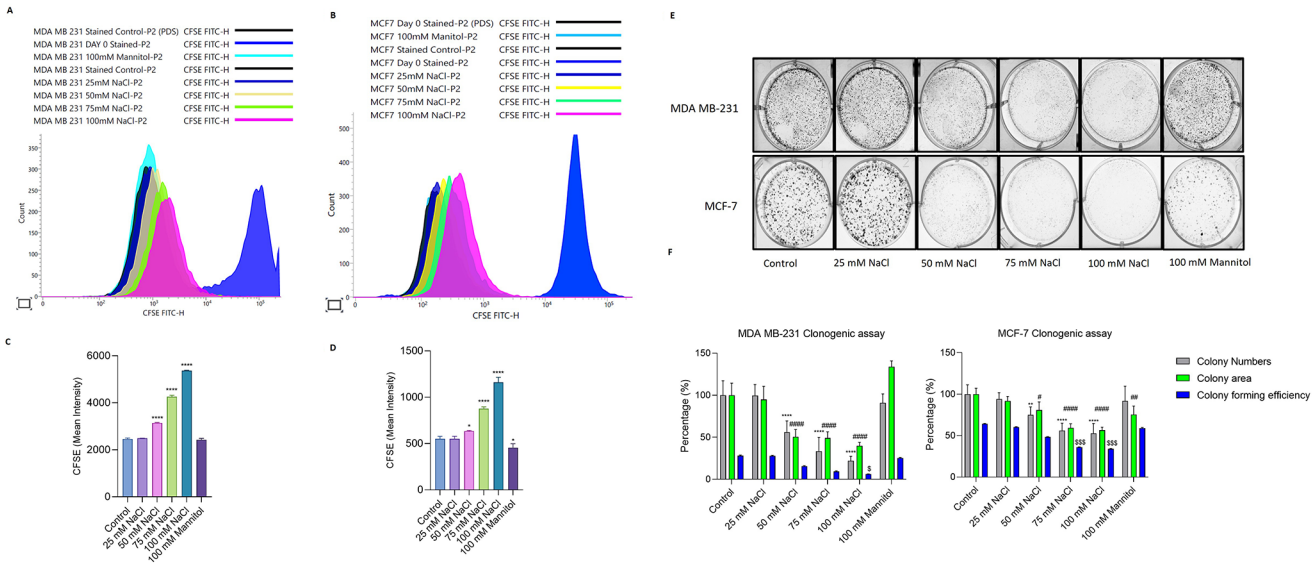


**Fig. 2** High salt inhibits cell proliferation and clonogenic properties in breast cancer cell lines. Cell proliferation assay of **A**. MDA MB-231 and **B**. MCF-7 cell lines under high salt conditions using CFSE dye dilution method in flow cytometry. **C** and **D**. Quantitative representation of CFSE intensity compared to DAY 0 control of MDA MB-231 and MCF-7 respectively. **E**. Clonogenic assay of MDA MB-231 and MCF-7 cell lines under different concentrations of NaCl. **F**. Quantitative representation of percent colony number, colony area, and colony forming efficiency of MDA MB-231 and MCF-7, respectively. (Statistical analysis by one-way ANOVA test. **, ##, $$ $p < 0.05$, ***, ###, $$$ $p < 0.005$, ****, ####$p < 0.0005$)

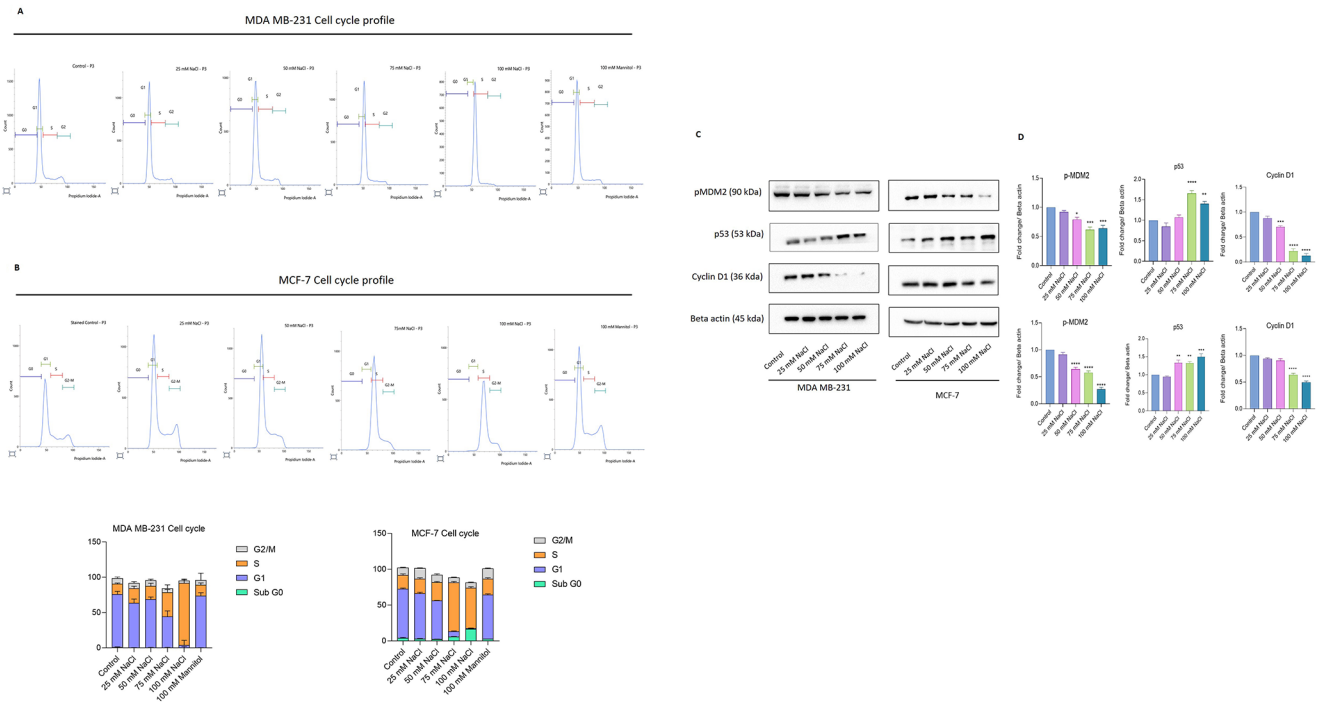


**Fig. 3** High salt induces cell cycle arrest in the G1/S phase in breast cancer cell lines. Cell cycle analysis of **A**. MDA MB-231 and **B**. MCF-7 cell line using PI in flow cytometer. **C**. Western blot analysis of different cell cycle markers. **D**. Densitometric analysis of blots using ImageJ software, NIH. (Statistical analysis by one-way ANOVA test. * $p = 0.02$, ** $p < 0.05$, *** $p < 0.005$, ****$p < 0.0005$)

24 and 48 h. (Fig. 4A-B). Our findings suggested that, compared to the control cells, salt treatment suppressed the migration of cells at both time points, whereas, mannitol exhibited no change in migration (Fig. 4C-D). The ability of cancer cells to adhere to extracellular matrix is another hallmark of metastatic behavior. Upon treatment with high salt, both MDA-MB-231 and MCF7 cells showed a significant reduction in adhesion to collagen I

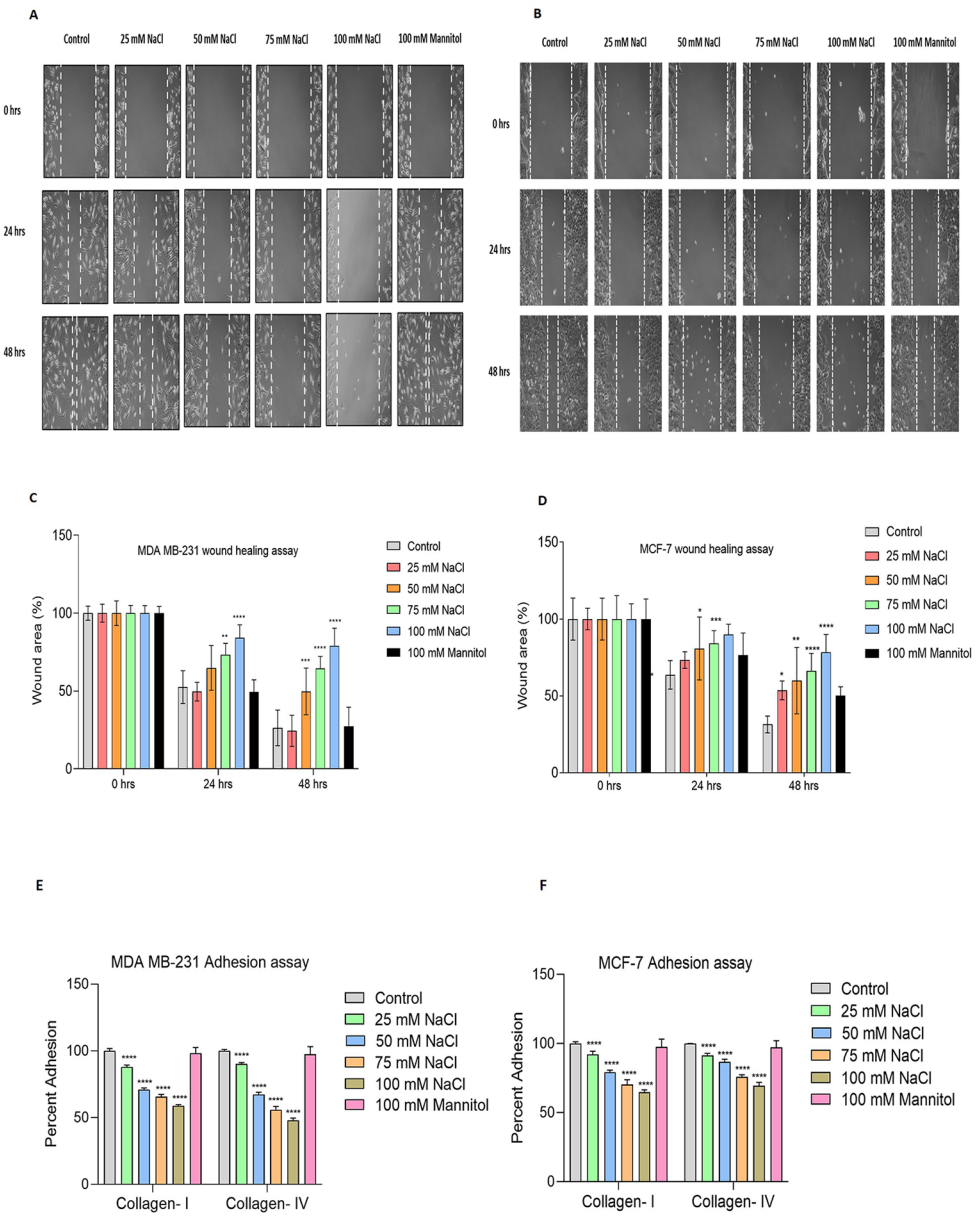


**Fig. 4** High salt suppresses the migration and adhesion in breast cancer cells. Wound healing assay of **A**. MDA MB-231 and **B**. MCF-7 cell lines under high salt conditions. **C**. and **D**. Quantitative representation of migrated cells at 0, 24, and 48 h. Adhesion assay of **E.** MDA MB-231 and **F**. MCF-7 cells under high salt stress condition (statistical analysis by two-way ANOVA test. * $p < 0.05$, ** $p < 0.005$, ***$p < 0.0005$)

and IV-coated wells (Fig. 4D-E). On the contrary, cells treated with mannitol did not affect the adhesion. These observations confirmed that the reduction in migration and adhesion in breast cancer cells is specifically attributable to salt rather than a change in osmolarity.

## Effect of high salt on global transcriptome profile of MDA MB-231 cells

High salt-treated MDA MB-231 cell line was subjected to RNA-Seq analysis for studying the global transcriptomic changes [GEO Submission (GSE265934)]. We identified 628 no of differentially expressed genes (DEGs) with p-value $< 0.05$ and absolute Log2 fold change of 1.5, which is represented as a volcano plot (Fig. 5A). Counts per million of top 50 genes sorted by P value were plotted as a heatmap, followed by Z-score normalization (Fig. 5B). Out of the 628 DEGs, 506 genes were upregulated and 122 were downregulated. To assess the potential functions of the 628 genes, overrepresentation analysis (ORA) using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) were conducted. Our analysis revealed that DEGs are significantly enriched in multiple KEGG pathways (Fig. 5C.) Notably, many of the enriched pathways were involved in cancer, such as PIK3-AKT (hsa04151), cell adhesion (hsa04514), calcium signaling (hsa04020), breast cancer (hsa05224), mTOR(hsa04150), MAPK (hsa04010), Ras (hsa04014), focal adhesion(hsa04510) etc. A complete list of KEGG pathways is given in Supplementary Table

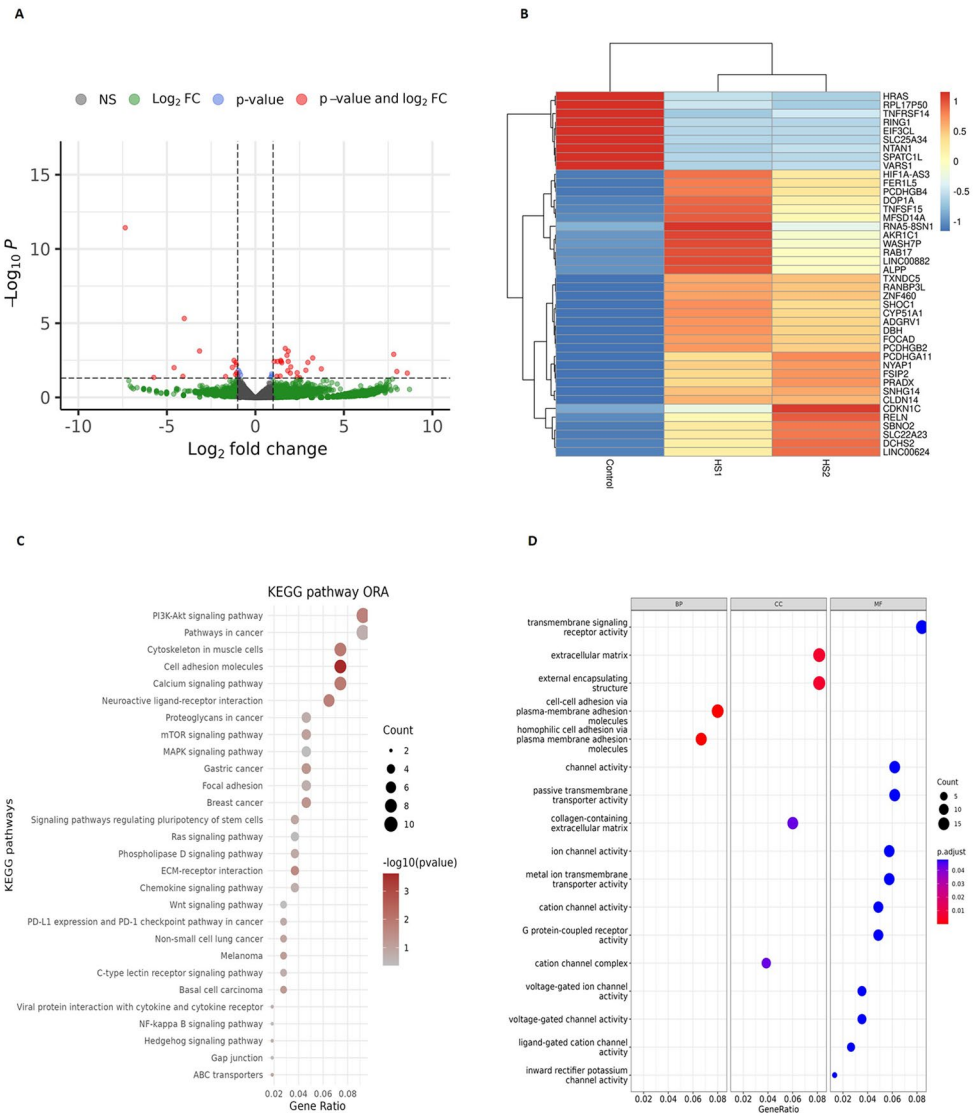


**Fig. 5** Differential gene expression analysis by RNA-sequencing under normal and high salt treatment conditions. **A**. Log2fold change and p-value of genes shown as a volcano plot. Red points represent differentially expressed genes that have p-value < 0.05 and absolute log2foldchange > 1.5. **B**. Z-score normalized counts per million reads of top 50 differentially expressed genes sorted by the p-value represented as heatmap. Samples are sorted by hierarchical clustering. **C**. Significant KEGG pathways that are enriched in differentially expressed genes **D**. Gene ontology overrepresentation analysis of differentially expressed genes (DEGs). The X axis represents gene ratio and; the Y axis represents enriched gene ontology terms

**Table 1** Differential gene expression data from RNA sequencing categorized by biological function based on literature analysis

| Biological Functions | Genes |
|---|---|
| Apoptosis | RANBP3L, SLC25A34, THBS2, CDON, PLCB2, EGR3, CDKN1C, KCNJ15 |
| Cell-cycle | CDKN1C, KCNJ15 |
| Metastasis | FOCAD, PCDHGA11, VARS1, RELN, THBS2, ICOSLG, LRRC4, NFASC, GNRHR, HERC2, KCNJ15, ANGPTL1 |
| Proliferation | EIF3CL, RING1, RANBP3L, FOCAD, PCDHGA11, CLDN14, RELN, ICOSLG, LRRC4, NFASC, HRAS, AKT3, LPAR6, KCNJ15, EFR3, GNRHR, KCNJ15 |

2. Gene ontology ORA of DEGs showed genes involved in transmembrane signaling receptor (GO:0004888), extracellular matrix (GO:0031012), cell-cell adhesion (GO:0098742), GPCR (GO:0004930), and cation channel genes (GO:0005261) (Fig. 5D). High salt treatment resulted in the up-regulation of DEGs with tumor suppressor or anti-cancer activity, while those with pro-tumorigenic functions were down-regulated. This could explain the anti-cancer effect of high salt in the breast cancer cell lines observed in our study. We compiled a gene list from the differentially expressed genes (DEGs) relevant to tumorigenesis. A comprehensive list of DEGs, along with their expression patterns and functions, is presented in Table 1. For validation of RNA-Seq data, mRNA expressions of 8 DEGs e.g. PCDHGA11, EIF2CL, RAVER1, TNFSF15, RANBP3L, MT1X, CLDN14, and CSF-2 were studied using qPCR. mRNA expressions of all these genes were in agreement with the RNA-Seq data (Fig. 6). Through a comprehensive literature review of genes enriched in our RNA sequencing data, we have identified genes potentially involved in the anti-cancer effects of high salt as evidenced by our experimental data. These genes are categorized based on their roles

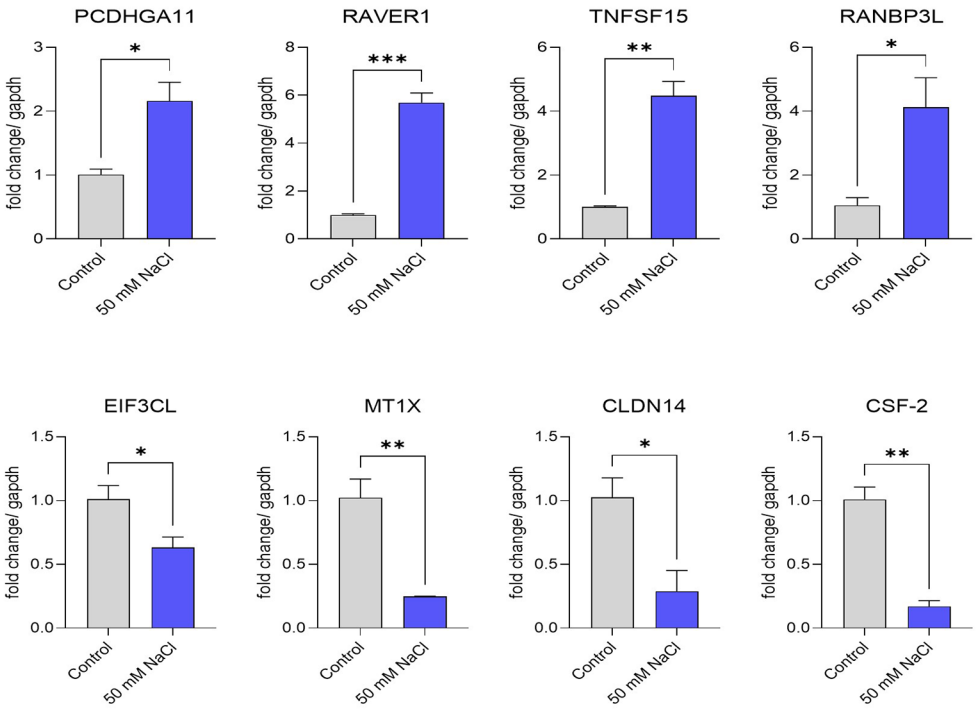


**Fig. 6** Real-time PCR-based validation of up and down regulated genes from RNA-Sequencing data in MDA MB-231 cell line under high salt stress conditions. (Statistical analysis by one-way ANOVA test. * $p = 0.01$, ** $p < 0.05$, *** $p < 0.005$, ****$p < 0.0005$)

in key biological processes such as apoptosis regulation, cell proliferation, metastasis, and cell adhesion (Table 1). A comprehensive list of top DEGs potentially involved in cancer, based on a literature review, along with their expression patterns and functions, is provided in Table 2.

## Discussion

The tumor microenvironment is a complex and dynamic ecosystem consisting of various cellular components, such as cancer cells, immune cells, blood vessels, and fibroblasts, as well as non-cellular elements like the extracellular matrix, cytokines, chemokines, and signaling molecules. This environment frequently undergoes various physiological and biochemical changes e.g. hypoxia, acidosis, increased interstitial fluid pressure, altered cytokine and chemokine profiles, extracellular matrix (ECM) remodeling, immune cell reprogramming and ionic imbalances. These imbalances play significant roles in the initiation, progression, and treatment outcome of cancer. One notable ionic imbalance in the tumor microenvironment is the misregulation of sodium ($Na^+$). In breast cancer patients, $^{23}$Na-MRI showed significantly higher sodium concentration in the tumor microenvironment compared to the surrounding healthy tissue [5, 6]. This raises questions about the impact of increased sodium concentration on tumor progression. In addition, the role of the extracellular concentrations of NaCl and their functional significance in tumors are poorly understood. In this study, we investigated the effect of high salt on breast cancer cell lines in vitro. Our study showed that treatment with NaCl sensitizes tumor cells towards apoptosis. To understand the molecular mechanism, we investigated the expression levels of pro and anti-apoptotic proteins. Our findings revealed that high salt suppresses the production of the anti-apoptotic protein Bcl-2 while promoting the expression of the pro-apoptotic protein Bax. The balance between these Bcl-2 family proteins is crucial in determining how a cell undergoes apoptosis from either intrinsic, extrinsic, or execution pathways [19]. In addition, Caspase 7 showed increased expression in both cell lines indicating the activation of the execution pathway of apoptosis. Reactive oxygen species (ROS), produced in cells through aerobic metabolism play an important role as regulators of signaling pathways involved in proliferation, differentiation, and survival [20]. A significant increase in intracellular ROS level triggers cancer cell cycle arrest, senescence, and apoptosis [21]. Breast cancer cells treated with high salt showed increase in ROS, which might be associated with the induction of apoptosis.

Mice fed with high-salt diet, showed reduced tumor size and metastatic foci compared to the control group. This outcome was linked to enhanced anti-tumor immunity, facilitated by the functional modulation of myeloid-derived suppressor cells (MDSCs) [20]. This modulation is involved in the decreased production of cytokines necessary for MDSC expansion, leading to MDSCs in the tumor microenvironment being primed towards an

**Table 2** List of selected DEGs with expression patterns and function in cancer. The screening criteria was based on the lowest false Discovery Rate (FDR) and fold changes exceeding 1.5-fold

| Sr. No | Gene Name | Up/ Down regulated in RNA-Seq Analysis | Function as per literature | References |
|---|---|---|---|---|
| 1. | EIF3CL | Down regulated | overexpression of EIF3F in human lung cancer cells alters cell proliferation and bioenergetics but also promotes metastasis in vivo. | [29] |
| 2. | RING 1 | Down regulated | RING1 depletion inhibited the proliferation and survival of the p53 wild-type cancer cells by inducing cell-cycle arrest, apoptosis, and senescence | [30] |
| 3. | HRAS | Down regulated | HRAS gene mutations contribute to cancer development by activating oncogenic signaling pathways, making HRAS a crucial player in tumorigenesis. | [31] |
| 4. | TNFSF15 | Up regulated | Tumor necrosis factor superfamily-15 (TNFSF15; also known as VEGI or TL1A) is a unique cytokine that functions in the modulation of vascular homeostasis and inflammation. Inhibits angiogenesis and helps reposition of macrophage phenotypes toward M1 by STAT1/3 activation. | [32] |
| 5. | RANBP3L | Up regulated | RanBP3's upregulation inhibits cell proliferation, induces apoptosis, and enhances drug sensitivity in CML cells by affecting the TGF-β-SMAD2/3-p21 axis and decreasing cytoplasmic ERK1/2 levels. | [33] |
| 6. | FOCAD | Up regulated | FOCAD acts as a tumor suppressor gene, with higher levels associated with better prognosis. In gliomas, FOCAD loss impacts cell proliferation and migration, affecting overall survival in patients | [34] |
| 7. | PCDHGA11 | Up regulated | It has been linked to regulating cancer cell proliferation and metastasis, acting as tumor suppressors by inhibiting these processes. Furthermore, the downregulation of PCDHGA11 in gastric cancer tissues has been associated with advanced tumor stages, relapse, and metastasis, highlighting its potential as an independent prognostic biomarker in cancer | [35] |
| 8. | VARS1 | Down regulated | The VARS1 gene plays a significant role in cancer progression and the tumor microenvironment. Studies have shown that VARS1 is a hub gene associated with M2-like macrophages in melanoma, promoting cell migration, invasion, and M2 macrophage polarization | [36] |
| 9. | CLDN14 | Up regulated | CLDN14 promoted the proliferation of CRC cells. | [37] |
| 10 | SLC25A34 | Down regulated | Inhibition of SLC25A34 may induce apoptosis (cell death) in cancer cells, making it relevant for cancer research. | [38] |
| 11 | RELN | Up regulated | RELN is involved in the regulation of invasion and proliferation of breast cancer. Moreover, RELN was found to inhibit both migration and invasion of pancreatic cancer cells | [39] |
| 12. | PCDHGB4 | Up regulated | PCDHGB4 played an important role in tumor immunity and confirmed that PCDHGB4 was associated with immune checkpoints, immune regulatory genes, and methyltransferases. Besides, enrichment analysis revealed that PCDHGB4 was involved in multiple cancer-related pathways | [40] |
| 13. | Rab17 | Up regulated | Rab17 might act as a tumor suppressor gene in hepatocellular carcinoma, and the antitumor effects of Rab17 might be partially mediated by the Erk pathway | [41] |
| 14. | CDKN1C | Up regulated | CDKN1C (encoding tumor suppressor p57KIP2) is a cyclin-dependent kinase (CDK) inhibitor whose family members are often transcriptionally downregulated in human cancer via promoter DNA methylation. | [42] |
| 15. | THBS2 | Up regulated | THBS2 expression is associated with PFI, immune cell infiltration, immune regulation, cell death, cell migration, epithelial–mesenchymal transition, angiogenesis and genomic variations in COAD | [43] |
| 16. | ICOSLG | Down regulated | ICOSLG knockdown inhibited the proliferation, migration, invasion and tumor formation of GC cells | [44] |
| 17. | LRRC4 | Up regulated | LRRC4/NGL-2 suppressed glioma cell proliferation by delaying the cell cycle in late G1 and invasion through regulating the expression of the invasion-related molecules including CD44, MMP16, TB10, and annexin A2 | [45] |
| 18. | NFASC | Down regulated | NFASC, along with NFIC, another member of the NF family, demonstrates potential as a tumor suppressor in lung squamous cell carcinoma (LUSC), inhibiting cell proliferation, migration, and invasion. | [46] |
| 19. | NCAM1 | Up regulated | In several human cancers, NCAM1 expression is downregulated. This suggests that it might have a tumor suppressor role. | [47] |
| 20. | HRAS | Down regulated | HRAS plays a crucial role in promoting cell growth and proliferation in cancer, particularly bladder cancer, making it a potential therapeutic target for inhibitors like salirasib. | [48] |
| 21. | AKT3 | Up regulated | Akt3 induces oxidative stress and DNA damage by activating the NADPH oxidase via phosphorylation of p47phox, leading to the upregulation of ROS levels and subsequent activation of the DNA damage response pathway. The DNA damage response plays a crucial role in inhibiting cell proliferation induced by Akt3 | [49] |

**Table 2** (continued)

| Sr. No | Gene Name | Up/ Down regulated in RNA-Seq Analysis | Function as per literature | References |
|---|---|---|---|---|
| 22 | LPAR6 | Up regulated | LPAR6 inhibits breast cancer growth via attenuating cell proliferation and acts as a tumor suppressor in breast cancer. | [50] |
| 23. | DEPTOR | Up regulated | Inhibits prostate tumorigenesis via the inactivation of mTORC1/2 signals | [51] |
| 24. | CDON | Up regulated | It is a well-known tumor suppressor and works by inducing apoptosis | [52] |
| 25. | PLCB2 | Down regulated | PLCB2 expression reduces melanoma cell viability and promotes melanoma cell apoptosis by altering Ras/Raf/MAPK signals | [53] |
| 26. | KCNJ15 | Up regulated | KCNJ15 inhibited cell proliferation and induced cell cycle arrest via upregulation of p21 protein expression. | [54] |
| 27. | CGNL1 | Down regulated | Cgnl1 function is crucial for sustaining neovascular growth and stability. | [55] |
| 28. | RIGI | Up regulated | RIG-I acts as a tumor suppressor in melanoma by regulating the activation of the MKK/p38MAPK signaling pathway | [56] |
| 29. | EGR3 | Up regulated | cell proliferation inhibition by Fas L expression and apoptosis induction via pro-apoptotic Bak and cell cycle inhibitor p21 expression. | [57] |
| 30. | GNRHR | Up regulated | GNRHR inhibits TNBC proliferation and metastasis, suggesting it could be targeted for TNBC treatment. | [58] |
| 31. | VARS1 | Down regulated | Multiple myeloma patients with abnormal high expression of VARS have a poor prognosis. VARS promotes the malignant growth of Multiple myeloma cells by affecting the regulation of valine metabolism. | [59] |
| 32. | HERC2 | Up regulated | HERC3 affect the migration, invasion and metastasis and further regulate EMT via EIF5A2/TGF-/Smad2/3 signal. | [60] |
| 33. | CDKN1C | Up regulated | Upregulation of CDKN1C resulted in the inhibition of hallmarks involving cell growth, differentiation, cell death, and angiogenesis in malignancies | [61] |
| 34. | KCNJ15 | Up regulated | KCNJ15 overexpression significantly inhibited RCC cell proliferation, migration, and colony formation, arrested the cell cycle and induced apoptosis of RCC cells in vitro. | [54] |
| 35. | ANGPTL1 | Up regulated | Angiopoietin-like protein 1 (ANGPTL1) is a member of the ANGPTL family that suppresses angiogenesis, cancer invasion, metastasis, and cancer progression in CRC | [62] |

immunostimulatory phenotype [21]. Alternatively, natural killer (NK) cell-mediated tumor immunity was also induced, by downregulating PD-1 expression while simultaneously boosting IFN-γ and serum hippurate levels [22]. Our study showcased that high salt independently hindered tumor proliferation, separate from the influence of immune cells as shown by earlier research. This was confirmed through the CFSE dye dilution assay and clonogenic assay. The inhibition of cell proliferation under high salt conditions is possibly due to increased sodium concentration impacting cellular activities or changes in the mechanisms that regulate the cell cycle. Our results also suggest that enhanced salt level suppressed the clonogenicity of the cells which might potentially affect proliferation at the primary tumor and newly formed metastatic sites.

High salt treatment induced distinctive cell cycle arrest at the G1/S phase as evident from flow cytometry analysis. Cyclin D1 also showed decreased expression following salt treatment. Previous studies have suggested that hyperosmotic conditions can induce cell cycle arrest through the downregulation of cyclin D1 and cyclin B1 expression [22], potentially leading to the arrest of the cell cycle at the G1/S and G2/M checkpoints [23]. Arrest of cell cycle at the G1/S checkpoint also depends on the activity of p53. Our study showed a decreased level of p-MDM2 and a subsequent increase in p53 expression following high salt exposure. In cells, the p53 level is regulated due to the action of MDM2, an E3 ubiquitin ligase, that targets p53 for degradation [24]. However, under certain physiological and genotoxic conditions, the level of p-MDM2 decreases, leading to the release of p53, which impacts cell cycle arrest and apoptosis [25]. This may be hypothesized that elevated salt concentrations within the tumor microenvironment could induce cell cycle arrest in proliferating neoplastic cells, thereby contributing to the inhibition of tumor progression.

Migration and adhesion of cells play a critical role during multiple stages of metastasis, encompassing local tissue penetration, entry into and exit from the bloodstream, and the re-establishment of cancer at distant organs [26]. Molecules associated with cell adhesion modulate tumor inhibitory actions and govern cellular movement via complex signaling networks and physical interactions in the tumor microenvironment [27]. In the metastatic process, various factors affect cancer cell migration. Among

these, cell detachment and adhesion to the extracellular matrix (ECM) at a new metastatic site, play a critical role in the formation of secondary tumors in distant organs [28]. High salt treatment inhibits the adhesion of breast cancer cell lines to collagen-I and IV matrices and markedly reduces migration efficiency of both the highly metastatic MDA MB 231 and the less aggressive MCF-7 lines. Therefore, high salt could potentially be utilized in therapeutic strategies aimed at inhibiting metastasis.

Our study from RNA-seq analysis demonstrates that high salt modulates the activity of 628 genes significantly across the control vs. high salt treated group. Subsequent KEGG enrichment analysis of these genes revealed their involvement in multiple cancer-related pathways, such as PI3K-AKT, MAPK, Cell adhesion, Ras, and calcium signaling pathway. Important genes from the KEGG pathway and GO ontology terms were selected based on their functional association in cancer. Eight such genes were validated using RT-PCR analysis. For example, KEGG ORA showed upregulation of PCDHGA11, a gene involved in cell-cell adhesion via plasma membrane, and is known to inhibit cancer cell proliferation and metastasis. Overexpression of EIF3CL which was reported to induce proliferation in human lung cancer cells, was downregulated under high salt treatment. Loss of FOCAD gene impacts cell proliferation and migration and affects overall survival in patients was found to be downregulated. CLDN14, an important gene in tight junction and promotes cancer cell proliferation, invasion, and migration by activating the PI3K/AKT/mTOR pathway was downregulated in high salt-treated cells. Downregulation of RanBP3L is reported to inhibit cell proliferation, induce apoptosis, and enhance drug sensitivity in CML cells by affecting the TGF-β-SMAD2/3-p21 axis. Our study showed significant upregulation of RanBP3L in the presence of high salt. The altered expression of these tumor suppressor genes in response to high salt could be further exploited in understanding the dynamics of cancer progression in complex tumor microenvironment and could be exploited for therapeutic of cancer treatment.

## Conclusion

In summary, our findings conclusively demonstrate that elevated salt levels induce apoptosis, impede cell proliferation, migration, adhesion, and arrest cell cycle progression. Most importantly, this is the first investigation that reveals the global transcriptomic profile of triple-negative breast cancer cell line, MDA MB-231 under high salt stress conditions. Differentially expressed genes (DEGs) and pathway analysis highlight the potential role of key mediators that are functionally associated with tumor suppression. These insights could be beneficial for the development of therapeutic strategies for cancer treatment.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s11033-024-09925-4.

**Author contributions** MS, AK, and RM: Conceptualization of the project. MS, UD, ASD, and KO: Designed the experiment. MS, UD, ASD, and KO Performed the experiment and analyzed the data. MS, UD, AK, and RM: Drafted the manuscript. AK and RM: Acquired the funds.

**Funding** RM[*1] acknowledge funding from the Science and Engineering Research Board, Govt. of India (CRG/2021/008212). MS[1] received Senior Research Fellowship from Indian Council for Medical Research (ICMR) (Sanction No.3/2/2/29/2022-NCD-III).

**Data availability** RNA-Sequencing NCBI GEO ID: GSE265934.

## Declarations

**Ethical approval** No ethical approval was required for this study.

**Competing interests** The authors declare no competing interests.

**Conflict of interest** Authors RM[*1], AK[*1], MS[1], UD[1], ASD[1], and KO[1] have no actual or potential conflict of interest.

**Supporting Information** Supplementary figure 1: ROS assay of high salt on MDA MB-231 and MCF-7 cell lines. Supplementary table 1: List of primer used in this study. Their sequences were designed using NCBI, PRIMER Blast tool. Supplementary table 2: List of all KEGG pathways from KEGG pathway over-representation analysis. Supplementary table 3: List of all Gene ontology terms from GO over-representation analysis.

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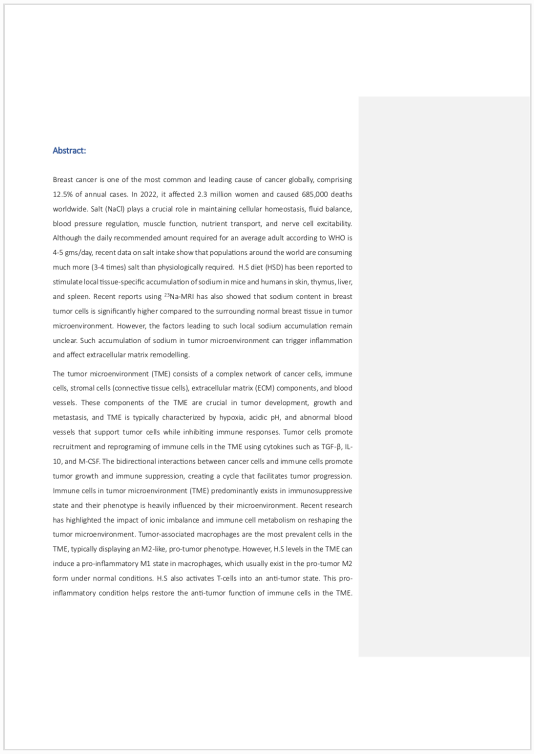

Abstract:

Breast cancer is one of the most common and leading cause of cancer globally, comprising 12.5% of annual cases. In 2022, it affected 2.3 million women and caused 685,000 deaths worldwide. Salt (NaCl) plays a crucial role in maintaining cellular homeostasis, fluid balance, blood pressure regulation, muscle function, nutrient transport, and nerve cell excitability. Although the daily recommended amount required for an average adult according to WHO is 4-5 gms/day, recent data on salt intake show that populations around the world are consuming much more (3-4 times) salt than physiologically required. H.5 diet (HSD) has been reported to stimulate local tissue-specific accumulation of sodium in mice and humans in skin, thymus, liver, and spleen. Recent reports using $^{23}$Na-MRI has also showed that sodium content in breast tumor cells is significantly higher compared to the surrounding normal breast tissue in tumor microenvironment. However, the factors leading to such local sodium accumulation remain unclear. Such accumulation of sodium in tumor microenvironment can trigger inflammation and affect extracellular matrix remodelling.

The tumor microenvironment (TME) consists of a complex network of cancer cells, immune cells, stromal cells (connective tissue cells), extracellular matrix (ECM) components, and blood vessels. These components of the TME are crucial in tumor development, growth and metastasis, and TME is typically characterized by hypoxia, acidic pH, and abnormal blood vessels that support tumor cells while inhibiting immune responses. Tumor cells promote recruitment and reprograming of immune cells in the TME using cytokines such as TGF-β, IL-10, and M-CSF. The bidirectional interactions between cancer cells and immune cells promote tumor growth and immune suppression, creating a cycle that facilitates tumor progression. Immune cells in tumor microenvironment (TME) predominantly exists in immunosuppressive state and their phenotype is heavily influenced by their microenvironment. Recent research has highlighted the impact of ionic imbalance and immune cell metabolism on reshaping the tumor microenvironment. Tumor-associated macrophages are the most prevalent cells in the TME, typically displaying an M2-like, pro-tumor phenotype. However, H.5 levels in the TME can induce a pro-inflammatory M1 state in macrophages, which usually exist in the pro-tumor M2 form under normal conditions. H.5 also activates T-cells into an anti-tumor state. This pro-inflammatory condition helps restore the anti-tumor function of immune cells in the TME.

# A Study on the Effect of Sodium Chloride on Breast Cancer Cells and Tumor-Associate Macrophages

*by* Manoj Sharma

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