Chapter VI: Conclusion and future prospects

6.1 Conclusion

Tumor microenvironment (TME) consists of stromal cells, immune cells, fibroblast cells, adipocytes, a network of lymph and blood vessels and an extracellular matrix [1]. TME is highly dynamic and undergoes constant change due to cross-talk between tumor cells and immune cells, cells undergoing apoptosis/necrosis, the presence of growth factors and metabolic changes [2]. Such dynamic changes cause TME to be hypoxic, acidic and high in metabolic wastes while there is a lack of nutrients due to the constant proliferation [3]. This led TME to be highly immunosuppressive causing secretion of factors from tumor cells and causing immune cells to be ineffective in carrying their normal function of killing damaged oncogenic cells [4]. This constant state of flux presents both challenges and opportunities for cancer therapy, as targeting components of the TME can influence tumor growth and response to treatment. Recent reports of ^{23}Na-MRI have revealed that the total sodium concentration (TSC) in solid tumors sodium is significantly higher compared to the surrounding healthy tissue [5-8]. Such high salt in tumor microenvironment is known to induce a pro-inflammatory state in macrophage, T cells, Treg cells and Dendritic cells [9-16]. High salt is also responsible for inhibiting the proliferation and expansion of myeloid-derived suppressor cells (MDSCs), which are responsible for inducing the immunosuppressive state in macrophage and dendritic cells [17]. Thus, high salt influences the pro-inflammatory state in the immune cells in tumor microenvironment potentially helping them in killing tumor cells. In literature, the role of high salt in cancer progression is still debated. Only limited reports suggest that high salt may exhibit anti-tumor properties through mechanisms such as inhibiting the accumulation and proliferation of MDSCs (myeloid-derived suppressor cells) and boosting the activity of NK (natural killer) cells within the tumor microenvironment [18-20]. However, the direct impact of high salt on cancer cells and its effects on the crosstalk between macrophages and cancer has not yet been investigated. To address this gap, the current study aimed to explore the effect of high NaCl on breast cancer cell lines and its impact on the cross-talk between macrophage/tumor-associated macrophage cells in vitro.

To investigate the effect of high salt (NaCl) on breast cancer cells, we conducted a series of experiments on two breast cancer cell lines: MDA-MB-231 (TNBC) and MCF-7 (ER+). Flow cytometry-based Annexin V and PI assay suggested that high salt treatment induced apoptosis significantly in both breast cancer cell lines in a concentration-dependent manner. This effect

was specifically due to NaCl and not changes in osmolarity, as other osmo-active agents (urea and mannitol) at similar concentrations did not induce apoptosis. Western blot analysis of apoptosis markers like Bax/Bcl-2 and Caspase 7 further confirmed the effect of high salt in the induction of apoptosis. Next, we examined whether high salt inhibits the proliferation of breast cancer cells using a CFSE dye dilution-based proliferation assay. The results showed that treatment with higher salt concentrations suppressed cell proliferation. This finding was confirmed by a clonogenic assay, which signifies the efficacy of cancer stem cells to proliferate and form colonies from single cells. Our results demonstrated that high salt suppressed the clonogenicity of both the MDA-MB-231 and MCF-7 cell lines. High salt treatment induced cell cycle arrest in the S phase, which was further validated by western blot analysis of cyclin-D1, pMDM2 and p53, proteins involved in cell-cycle arrest and apoptosis induction. Given that metastasis is a significant challenge in cancer treatment, we also investigated if high salt affects cell migration in MCF-7 and the highly metastatic MDA-MB-231 cell line. Wound healing assay suggested that high salt treatment suppressed cancer cell migration in a concentration-dependent manner. In contrast, cells treated with mannitol showed similar migration status as control cells, suggesting the specific role of high salt in inhibiting migration. Once metastasis is initiated, cancer cells migrate from the primary site and travel to a new location in order to establish a microenvironment conducive to cancer growth. The process involves the invasion of the extracellular matrix (ECM) and the binding to collagen components within the ECM through adhesion molecules. To investigate the impact of high salt on cell adhesion to the ECM, collagen I and collagen IV, the key elements of ECM were utilized. The results showed that cancer cells treated with high salt displayed reduced adhesion to collagen, suggesting the inhibitory effect of high salt on the attachment of breast cancer cells to the ECM. To understand the global transcriptomics changes and the pathways involved in high salt-treated triple-negative MDA MB-231 cells, RNA Sequencing analysis was performed. The analysis showed differential expressions of various genes involved in apoptosis, cell-cycle arrest, metastasis, and proliferation in response to salt treatment. KEGG pathway and GO ontology over-representation analysis (ORA), showed expression of genes involved in multiple cancer-related pathways, such as PI3K-AKT, MAPK, Cell adhesion, Ras, and calcium signalling pathway. Transcriptomic analysis of MDA MB-231 cells also showed increased expression of genes involved in tumor suppression.

Next, we explored the effect of high salt on inducing inflammation and its effect on the cross-talk between TAM and breast cancer cells. High salt induces pro-inflammatory cytokine production in THP-1 macrophage and tumor-associated macrophage (TAM) cells. High salt also induces MCP-1-mediated chemotaxis of monocytes toward MDA MB-231 cells. Increased infiltration of macrophage cells in tumor microenvironment is found to have a positive impact on cancer outcomes. However, most of the immune cells infiltrating TME under the influence of cancer cells are converted into an immunosuppressive state. High salt was found to suppress the formation of TAMs, which was evident from the changes in morphology and gene expression. To mimic the cross-talk between macrophage and breast cancer cells, we did a co-culture study between macrophage and breast cancer cells using a transwell insert under high salt treatment. THP-1 macrophages and TAM co-cultured under high salt stress conditions suppressed the proliferation of MDA MB-231 cells exemplifying the critical role of salt on both cancer cells and TAMs. Macrophages and TAMs inhibited the migration and invasion of highly metastatic MDA MB-231 cells under high salt conditions. They also inhibited the adhesion of MDA MB-231 cells on collagen-I and collagen- IV coated extracellular matrix (ECM). Further, RNA-sequencing analysis of MDA MB-231 co-cultured with TAM, showed genes enriched in ribosome biogenesis, oxidative phosphorylation, cellular respiration and ATP synthesis. High salt treatment suppressed genes related to ribosomes, respiration, and cellular energetics. Ribosomes are crucial for protein synthesis, particularly in rapidly proliferating tumor cells, and recent studies have highlighted additional, non-traditional roles for ribosomes in cancer. Our study found downregulation of several ribosomal proteins such as RPS12, RPS27L, RSL1D1, RPS13, RPS4X, RPL21 and RPL23 which are known for their anti-cancer functions. Additionally, multiple genes functioning as tumor suppressors were upregulated in MDA-MB-231 cells. These findings could potentially explain the anti-cancer efficacy of high salt in the presence of TAMs.

Overall, from our study it may be concluded that high salt induces anti-tumor effect in breast cancer cells by suppressing proliferation, metastasis, adhesion, clonogenicity and inducing apoptosis. High salt also induces increased infiltration of monocyte cells towards the site of cancer. High salt induces a pro-inflammatory state in infiltrating macrophages and tumor-associated macrophage (TAM) cells *in vitro*. High salt showed upregulation of genes possessing anti-tumor functions. Co-culture study with macrophage/TAMs showed increased suppression

of proliferation, migration, invasion and adhesion of MDA MB-231 cells in the presence of high salt. Further RNA Sequencing analysis showed upregulation of genes known to function as tumor suppressor genes and those involved in inhibiting cell proliferation, metastasis, cell cycle and apoptosis.

6.2 Future Prospects

Our data confirmed the anti-tumorigenic role of high salt on breast cancer cells. However, we propose further research works that can be taken up to understand the effect of high salt on breast cancer cells.

1. To understand if sodium accumulation is a global phenomenon specifically in solid tumors, the effect of high salt should be studied and validated in other solid tumors, such as lung cancer, colon cancer, and brain cancer using sophisticated techniques like ^{23}Na MRI.

2. Cell lineage tracing can be used to accurately determine the origin of tumor-associated macrophage cells in the tumor microenvironment. This technique will help to understand the effect of high salt on infiltrating macrophage cells and tissue-resident macrophages. Ultimately, this will provide insights into whether, within the complex and dynamic tumor microenvironment, high salt can influence cancer cells to immunosuppress infiltrating macrophages and tissue-resident cells.

3. Performing a global analysis of secretory factors in the TME of solid tumors under high salt conditions will provide a dynamic view of the pro-inflammatory state of immune cells in the TME. An LC-MS/MS-based approach will help us identify and specifically target pathways to improve cancer treatment outcomes.

4. Our RNA sequencing experiment has revealed several genes that express differentially in breast cancer cells under high salt stress. The function of selected DEG's can be confirmed in breast cancer cell lines as well as in mice models using siRNA or CRISPR-based knockout under high salt conditions.

5. The effect of high salt on the metabolic state of breast cancer cells will provide us with details about the changes associated with cellular energetics, metabolism, cellular respiration, mitochondrial function, and ribosome activity. This can help us to strategize further approaches to target cancer using metabolic inhibitors.

6.3 References

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