ENHANCEMENT OF MODULATED ELECTRO-HYPERTHERMIA EFFECTS BY HEAT SHOCK FACTOR 1 INHIBITION AND RE-SENSITIZATION OF CANCER CELLS TO ANTIPROGESTINS IN A TRIPLE-NEGATIVE BREAST CANCER MODEL

PhD thesis

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1. Introduction

Female breast cancer is the most prevalent type of cancer globally. Triple-Negative Breast Cancer (TNBC) is an aggressive type of breast cancer that does not express estrogen- and progesterone receptors, and HER2. Because TNBC tumors lack the receptors expression, they are not sensitive to endocrine therapy and efficient TNBC treatments are still missing.

The Heat Shock Response (HSR) is a fundamental cellular mechanism that plays a critical role in maintaining proteostasis and cellular homeostasis under stress conditions, and the Heat Shock Factor 1 (HSF1) is the master regulator of the HSR. Dysregulation of the HSR is known to play a critical role in cancer. In cancer cells, the activation of HSF1 often results in the abnormal upregulation of Heat Shock Proteins (HSPs), such as HSP70. This upregulation provides these cells with a selective advantage by promoting cell survival, inhibiting apoptosis, facilitating the development of aggressive phenotypes, and inducing thermotolerance.

Hyperthermia is a therapeutic approach that involves raising the temperature of a part of or the entire body to treat various medical conditions. In the context of cancer therapy, hyperthermia involves heating the cancerous area to enhance the effectiveness of treatments like chemotherapy and radiotherapy.

Modulated electro-hyperthermia (mEHT) is a non-invasive hyperthermia therapy, using an amplitude-modulated 13.56 MHz radiofrequency electromagnetic field to induce tumor cell destruction at 42 °C. The mechanisms underlying mEHT involve a combination of thermal and non-thermal effects, and their synergism results in activation of apoptotic pathways. However, mEHT can induce HSR, and the heat-induced thermotolerance can protect cancer cells from hyperthermia-induced apoptosis. In turn, silencing or inhibiting HSF1 through gene-editing techniques or small molecule inhibitors like KRIBB11, a specific HSF1 inhibitor that directly blocks HSF1 transcriptional activity, has illuminated the potential to reverse thermotolerance in cancer cells. This approach may enhance the efficiency of hyperthermia treatments against cancer cells.

The Progesterone Receptor (PGR), a nuclear receptor activated by progesterone, has two key isoforms, PRA and PRB. The PRA/PRB ratio impacts breast cancer outcomes, with elevated PRA levels linked to poorer prognosis and shorter disease-free survival, but plays a significant role in the response to endocrine therapy using Selective Progesterone Receptor Modulators (SPRMs), while PRB mediates cell proliferation. SPRMs or antiprogestins are a new class of compounds developed to target the PGR. This group includes mifepristone (MIF) and ulipristal acetate (UPA), that in breast cancer are responsible for increase apoptosis and decrease cell proliferation.

2. Objectives

We aimed to investigate the effects of HSF1 downregulation by CRISPR/Cas9 technology subsequent to mEHT treatments *in vivo* and to check for the translational potential of HSF1 inhibition by KRIBB11 in combination with mEHT at the mRNA and protein levels. Moreover, we aimed to elucidate the alterations on PGR expression in response to *in vivo* mEHT treatments at the mRNA and protein levels, and to check the potential synergistic effect

of combining mEHT with MIF or UPA on the viability of TNBC cells.

3. Materials and Methods

Cell viability assay: The 4T1 murine mammary carcinoma cell line was used for both *in vitro* and *in vivo* studies. The viable yield was determined by resazurin assay method. Cells were seeded into 96-well plate at a density of 5 x 10^3 cells/well, resazurin was added and incubated for 2 hours in humidity chamber (5% CO_2 at 37°C). Fluorescence was recorded at 560 nm excitation / 590 nm emission filter set using a microplate reader.

HSF1 construct: CRISPR Guide RNA (gRNA) Lentiviral Transduction particles was used for knockdown of HSF1. Cells were seeded in a 96-well plate, treated with hexadimethrine bromide for transduction enhancement, then exposed to lentiviral particles. Selection with puromycin identified successfully transfected cells, confirmed by fluorescence microscopy. Positive cells were FACS-sorted into 4T1 wild type, empty vector, and HSF1-KO groups. Flow cytometry checked GFP-positivity.

***In vivo* HSF1 inhibition model:** 6-8 week old female BALB/c mice were inoculated with either 4T1 wild type (WT) cells, or empty vector (EV) cells, or HSF1-KO (KO) cells into the mammary fat pad under isoflurane anaesthesia. Mice were randomized into treatment groups according to their tumor volume at day 8. For KRIBB11 experiment, KRIBB11 was administrated intraperitoneally at a dose of 50 mg/kg/day for 8 days. mEHT treatments were performed every two days and tumor volume was monitored by ultrasound and caliper between mEHT treatments. The study was terminated on day 16 with the harvest of tumors.

***In vivo* mEHT treatments:** Tumors were mEHT-treated four times for 30 minutes plus 5 minutes for device stabilization with applied energy that varied between 0.2 and 1.0 watts. Temperature monitoring was performed with optical sensors. For Sham treatments, cables were disconnected, therefore, no electromagnetic field was generated, and no energy was transferred (no heating).

***In vitro* mEHT treatment:** WT 4T1 cell suspension was treated with LabEHY200 *in vitro* applicator. Treatments

were performed in a temperature-driven way, maintaining 42 °C in for 30 minutes. The cells were incubated with either MIF or UPA at 32 or 35 μM, respectively, or 0.01% DMSO. After 24 hours, cell viability was assessed using the resazurin assay.

Histopathology and immunohistochemistry (IHC): Formalin-fixed, paraffin-embedded tumor tissue samples were sectioned and mounted on glass slides for Hematoxylin and Eosin (H&E) staining to assess tumor cell destruction (TDR), calculated as the ratio of damaged area to the entire tumor area. Additionally, Immunohistochemistry (IHC) analysis included markers for HSF1, HSP70, and PGR expression. The IHC protocol included steps for antigen retrieval, endogenous peroxidase blocking, non-specific protein blocking, antibody incubation, and visualization of the immune reaction. Slides were digitally scanned and assessed using CaseViewer software. The intensity of the immune reaction, quantified as the relative mask area ratio (rMA), was used for analysis.

RNA isolation and real-time PCR (RT-PCR): RNA was isolated with the TRI reagent® RT. Reverse transcription

of isolated RNA was performed by High-Capacity cDNA Reverse Transcription Kit. The amplified cDNA was used as a template for RT-PCR. The following gene expressions were measured: HSF1, HSP70, and PGR. Gene expression was normalized to GAPDH or RPLP0.

Next-Generation Sequencing (NGS) and Bioinformatics Analysis: RNA integrity and RNA concentration were assessed by the RNA ScreenTape system with the 2200 Tapestation and the RNA HS Assay Kit with the Qubit 3.0 Fluorometer. Library preparation and sequencing followed standard protocols. Reads were aligned to the Mus musculus reference genome. Differential expression (DE) analysis used FC > 2.0 and p-value < 0.05 thresholds. Uninformative genes were excluded based on literature search. Remaining DE genes were grouped into functional categories.

NanoString analysis: Total RNA was hybridized to the customized nCounter® gene panel. The applied custom gene panel was composed by 134 genes, including PGR, identified as differentially expressed by NGS. Samples were transferred to the nCounter Prep Station for further

processing. The gene expression profiles of the samples were digitalized with the nCounter Digital Analyzer. Quality assessment and normalization were performed in nSolver 4.0 Analysis Software.

Statistical analysis: Statistical analyses were performed using the GraphPad Prism software. Differences between groups were assessed using the following methods: One-way ANOVA for comparisons involving more than two groups, Two-way ANOVA for longitudinal measurements (such as tumor volume), and t-tests for comparing sham-treated and mEHT-treated groups. Differences were considered statistically significant at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns = not significant. Data are given as mean ± Standard Deviation (SD).

4. Results

Successful transduction of 4T1 cell line with the HSF1-gene editing lentiviral construct: Flow cytometry was used to assess the knockdown efficiency of our model. As expected, non-transduced WT 4T1 cells did not express GFP. In contrast, more than 95% of the sorted cells in the HSF1-KO group were GFP+, meaning that these cells took

up the lentiviral construct. Transduction with the EV was less effective, resulting in 62.8% of cells being GFP+.

Successful reduction of HSF1 and HSP70 expression in transduced TNBC cell line: Subsequent treatment with conventional hyperthermia *in vitro* showed significant downregulation of HSF1 gene expression in the knock-down group, and the heat induced stimulation of HSP70 decreased in the HSF1-KO group at 42°C.

mEHT-induced tumor growth reduction was enhanced in HSF1-KO tumors: Sham EV tumors doubled in volume, while Sham HSF-1 KO tumors were smaller. mEHT-treated tumors showed reduced growth, with HSF1-KO mEHT group having the slowest growth. Tumor volume and mass were notably smaller in mEHT KO group. Tumor size correlated with mass consistently.

mEHT-induced tumor destruction was enhanced in HSF1-KO tumors: TDR was minimal in sham tumors, but significantly increased in mEHT-treated tumors. The tumor damage induced by mEHT was further increased in the mEHT-treated HSF1-KO group, with the difference not reaching statistical significance.

HSF1-KO prevented HSF1 and HSP70 upregulation after mEHT treatment *in vivo*: qPCR and IHC data revealed a significant reduction in HSF1 expression in the KO groups, suggesting effective silencing. mEHT did not change HSF1 expression in the KO group, as observed in the EV group. Likewise, HSP70 expression, upregulated by mEHT in the EV group, was not significantly induced by mEHT in the KO group.

mEHT-tumor growth reduction was synergistically enhanced by KRIBB11 following mEHT treatments: A synergistic effect was observed with the combined treatment of mEHT and KRIBB11. The combination group showed a significant decrease in tumor growth rate compared to mEHT alone. Additionally, tumor mass data indicated a substantial reduction in the mEHT-treated KRIBB11 group. However, KRIBB11 monotherapy did not reduce tumor growth.

Slightly increased tumor destruction tendency was observed in tumors treated with combined therapy: TDR revealed significant tissue damage in mEHT + KRIBB11-treated tumors compared to Sham. A negative correlation

between TDR and tumor mass was observed. In general, mEHT-treated tumors were smaller and showed bigger TDRs. In turn, larger sham tumors tended to have moderate TDRs.

KRIBB11 prevented HSP70 upregulation after 4 mEHT treatments: qPCR and IHC data revealed no significant reduction in HSF1 expression with KRIBB11 compared to the vehicle. There were no significant differences between the Sham and mEHT KRIBB11 groups. In contrast, HSP70 levels tended to increase with mEHT + vehicle treatment, while the combination of mEHT + KRIBB11 led to a significant reduction in HSP70 upregulation.

Analysis of mEHT effects on gene expression revealed upregulation of PGR expression: NGS revealed that PGR demonstrated significant upregulation in the mEHT-treated group (FC = 16.05; p value = 0.01) in the 4T1 TNBC mouse model. For validation of gene expression at the mRNA level, individual mRNA molecular counting was performed with NanoString. Again, PGR was significantly upregulated in the mEHT-treated group. PGR

mRNA levels were further analyzed by qPCR. Compared to sham group, the mRNA expression of PGR gene was significantly upregulated after mEHT treatments.

mEHT upregulated PGR protein expression in TNBC malignant tumors: To confirm the re-expression of PGR by mEHT treatments, PGR expression was assessed by IHC. Consistent with the multiplex analysis and qPCR, the percentage of relative PGR masked area significantly increased in mEHT-treated malignant tumor samples compared to sham group.

Increased sensitivity to MIF and UPA in combination with mEHT in TNBC cells: The potential to re-express PGR in TNBC cells offers an important avenue for re-sensitizing these cells to SPRMs. MIF or UPA reduced cell viability at 37°C. Conventional hyperthermia did not significantly enhance the cell killing effect of both drugs. However, the combination of MIF or UPA with mEHT resulted in a significant decrease in cell viability, suggesting enhanced cell-killing effects compared to individual treatments.

5. Conclusions

In this study, we investigated the combined effects of mEHT with HSF1 knockdown and the specific HSF1 inhibitor, KRIBB11, on inhibiting tumor growth in a TNBC mouse model. We also investigated the potential re-activation of PGR in the 4T1 TNBC mouse model following mEHT treatments, and whether this re-activation sensitizes TNBC cells to antiprogestins, MIF or UPA. We can further conclude that:

- CRISPR/Cas9-mediated HSF1 knockdown was successful and exhibited high transfection efficiency in 4T1 murine TNBC cells.
- The mEHT cancer cell-killing effect was enhanced by the knockdown of HSF1.
- Integration of KRIBB11 alongside mEHT demonstrated a synergistic effect with significant reduction of tumor growth.
- HSF1 inhibition, either by CRISPR/Cas9 or KRIBB11, resulted in a diminishment of HSP70

upregulation typically seen after mEHT treatments.

- The multiplex analysis and qPCR revealed the re-establishment of PGR expression in 4T1 TNBC mouse model treated with mEHT.
- The re-expression of PGR was also confirmed in the protein level.
- The combination of mEHT treatments and antiprogestins, MIF or UPA, reduced 4T1 cell viability in vitro, resulting in additional cell-killing effect.
- Conventional hyperthermia did not enhance the cell-killing effect of MIF or UPA.

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