MULTIPLEX ASSESSMENT OF MODULATED ELECTRO-HYPERTHERMIA INDUCED LOCAL ACUTE PHASE PROTEIN PRODUCTION IN A MOUSE TNBC MODEL

PhD thesis

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1. Introduction

Hyperthermia is applied in oncological treatments as a complementary treatment modality, beneficially enhancing the therapeutic effects of chemo-, radio- and immunotherapy. Oncological hyperthermia means the heating of malignant tissues up to 40-48 °C.

Hyperthermia induce several biological processes in tumors at the tissue, cellular and molecular level. At tissue level, hyperthermia increases the initially high metabolic rate of the tumor, resulting in insufficient oxygen and nutrient supply and a consecutive hypoxia and acidosis. Additionally, blood flow of the tumor decreases, by heating over 42 ^{o}C. At the cellular and molecular level, hyperthermia induces denaturation of DNA repair proteins and consequent DNA damage, heat shock response (HSR), cell cycle arrest and cell death, primarily apoptosis.

Hyperthermia can synergize with classical oncological treatments: while both chemo-and radiotherapy are effective in the well-oxygenized regions of the tumor and exert cell-cycle inhibitory effects in the M and G phase, hyperthermia is efficient in low-oxygenized regions and blocks cell cycle mostly in the S phase.

Modulated electro-hyperthermia is a local, non-invasive deep hyperthermia method, using a capacitively coupled, electromagnetic field (EMF) in the radiofrequency range (13.56 MHz). It utilizes the difference of malignant and healthy tissues, regarding bioelectrical

properties. Cancer cells have altered metabolism, called the Warburg effect, which results in elevated ion, lactate and H^+ concentration. Therefore, tumor tissues have higher permittivity (ε) and conductivity (σ) than healthy tissues. Moreover, the water content of the tumor tissue is also increased. All these factors result in decreased impedance (Z) and the enrichment of the electromagnetic field's (EMF) energy, allowing a selective heating even in deeply located tumors. Besides the thermal effects, EMF have non-thermal effects, such as rotation of cells, electro-permeabilization and electroporation. Thermal and non-thermal processes augment each other's tumor cell killing effects.

Breast cancer is the most common malignancy type and accountable for the most cancer-related deaths among women worldwide. Breast cancers can be categorized according to the expression of progesterone (PR), estrogen receptor (PR), Human Epithelial Growth Factor receptor (HER2) and Ki67. Triple Negative (TNBC; ER-, PR-, HER2-, Ki67↑) subgroup is a markedly aggressive subtype with high chance of recurrence. In non-TNBC patients, primary treatment contains anti-hormonal therapy, while in TNBC, this option is unavailable.

For preclinical investigation of TNBC, 410.4-derived mouse mammary carcinoma cell lines were used in our studies. The most aggressive, 4T1 cells generate immune-desert tumors with high metastatic potential, while 4T07 are less aggressive, creating tumors with higher immunogenicity. Implantation of these cells into mammary fat pad of

BALB/c mice generates isografts providing a reliable in vivo model of TNBC.

Cellular stress response is an adaptive mechanism of cells to environmental stressors, like heat, radiation, oxidative stress, etc. One of the most well-known adaptive mechanisms is the intracellular heat-shock response (HSR), which can support the repair or elimination of damaged proteins. The HSR can be suppressed by the inhibition of Heat shock factor-1 (Hsf-1). KRIBB11 is a novel, selective Hsf-1 inhibitor.

Acute Phase Reaction (APR) is traditionally considered as the non-specific reaction of multicellular organisms against harming stimuli like infection, tissue injury or chronic diseases. Acute phase proteins (APP) are produced by the liver and released into the circulation as part of the systemic humoral inflammatory response in different conditions, like chronic diseases or cancer. However, APPs can be produced by non-hepatic cells, like immune-, epithelial-, and other parenchymal- (such as renal epithelial)- cells, astrocytes, etc. and can exert local, non-canonical functions. Cancer cells can also produce APPs. Most of the APPs function as tumor-protecting factors extracellularly in the Tumor Microenvironment (TME). Complement factors can contribute to local, tumor-promoting imflammation and stimulate angiogenesis without the activation of complement cascade. Fibrinogens can promote tumor growth by serving as a reservoir for growth factors and defensive barrier against anti-tumoral immune cells. Protease inhibitors (Serpins and

Itihs) can neutralize proteases, produced by immune cells and haptoglobin participates in the metabolic reprogramming of cancer cells and protection against oxidative stress.

2. Objectives

We aimed to investigate the tumor growth-inhibiting effects of mEHT in TNBC mouse model. We aimed to reveal the most relevant molecular effects and pathways induced by mEHT in treated tumors with a comprehensive, multiplex analysis at mRNA and protein level.

3. Materials and Methods

Tumor model and experimental design: Female, 6-8 week old BALB/c mice were inoculated in the mammary fat pad under isoflurane anesthesia to create isografts. 4T1 cells were used for the short-and medium term and 4T07 cells for the long-term experiments. Six days after inoculation, mice were randomized by tumor size and final tumor size measurement was performed on the final day, when the experiment was terminated with sacrifice of the mice and removal of the tumors. mEHT treatments were performed during the experiments every 48 hours. Tumors were harvested 24 or in the case of the time-kinetic experiments, 4, 12, 24, 48, 72 hours after the last treatment. Tumor weights were measured and samples were stored for histological and molecular analysis.

In vivo treatments: Tumors were treated for 30 minutes with the LabEHY200 modulated electro-hyperthermia (mEHT) device. Single or repeated - 3 × (medium) or 5 × (long) treatment was applied in separate experiments. Tumors were exposed to a capacitively coupled, radiofrequency electromagnetic field, established between the lower (applicator, heating pad) and the upper electrode (pole). Treatments were performed in a power-adjusted, temperature-driven way: skin temperature above the tumor was kept at 40 °C – ensuring 42 °C in the tumor; rectal, heating pad and room temperature was monitored. Control animals received sham treatment.

In vitro treatments: 4T1 cell suspension in a plastic bag was treated with LabEHY200 in vitro applicator. Treatments were performed in a temperature-driven way, maintaining 42 °C in the bag for 30 mintues. Cells were collected and lysed with Tri-Reagent for RNA extraction. KRIBB11 or DMSO (control) treatments were performed 1 hour before mEHT treatment in the combination experiments.

Histopathology and immunohistochemistry: Formalin-fixed, paraffin-embedded tumor tissue samples were cut and mounted on glass slides for H&E staining and immunohistochemistry (IHC for Hsp70 and cC3). Immunohistochemistry protocol contained antigen retrieval, endogenous peroxidase, non-specific protein blocking, incubation with

antibodies and visualization of the immune-reaction. Slides were scanned and evaluated digitally with CaseViewer software. Intensity of the immune reaction, expressed as relative mask area as a ratio of the living area was used for quantification. Tumor Destruction Ratio (TDR%) was calculated on H&E and cC3 stained slides.

RNA isolation and real-time PCR: RNA was isolated using Tri-Reagent. Reverse transcription was performed by High-Capacity cDNA Reverse Transcription Kit. After amplification cDNA was used as template for RT-PCR. The following gene expressions were measured: Complement component 4b (C4b), Haptoglobin (Hp), Apoptosis Inducing Factor (AIF). Gene expression was normalized to 18S expression.

Next-Generation Sequencing (RNA Seq) and Bioinformatical Analysis: RNA integrity and concentration were determined with Qubit 3.0 Fluorometer and RNA ScreenTape system. After removal of rRNAs, DNase treatment were performed and RNA-Seq libraries were prepared. Quality and quantity of the libraries were determined and pooled libraries were diluted for 2 × 80 bp paired-end sequencing with NextSeq 550 Sequencing System. Reads were aligned with Mus musculus (GRCm38) reference genome and associated with known protein-coding genes. Number of reads aligned within a gene was

quantified and normalized. Voom approach in the limma package was used for statistical analysis and counts were displayed as transcripts/million. FC > 2 and p-value < 0.05 were used to determine differentially expressed (DE) genes. An in silico analysis was performed with upregulated DE genes using the gene ontology (GO) database. Kendall tau's method was used to create a heat map from the normalized NGS RNA Seq data.

Mass Spectrometry Analysis: Liquid chromatography with tandem mass spectrometry analysis was performed. After sample separation mass analyzer was used for obtaining the full MS mass spectra of proteins in the range of 300 to 2000 m/z. MS/MS spectra were acquired by higher-energy collisional dissociation (HCD) fragmentation. Precursor ions that possessed charge states (>1) were selected for the MS/MS fragmentation. MaxQuant proteomics software was used for data analysis with Mus musculus in Uniprot database. Quantification of identified proteins was performed by the relative label-free quantification (LFQ) algorithm in MaxQuant.

Nanostring Analysis: We established a custom panel for validation based on the relevance in breast cancer and heat shock topic using the NGS data and literature search. Absolut RNA count was determined by bar-coding each specific RNA probe with colored labels. RNA

concentration of samples were examined by Qubit 4 Fluorometer and samples compassing adequate RNA amount were hybridized to the customized nCounter® gene panel, containing 134, DE genes, based on the NGS data and literature search. nCounter Digital Analyzer was used to digitalize gene expression profiles of the samples and nSolver 4.0 Analysis Software was used to quantify the results to get absolute RNA counts.

Statistical Analysis: The GraphPad Prism software was used for statistical analysis. Sham and mEHT treated groups were compared with parametric unpaired T-tests or nonparametric unpaired Mann-Whitney tests. One-way ANOVA was used for comparing more than two groups. Follow-up experiments were analysed with two-way ANOVA with Bonferroni correction. The null hypothesis was rejected if the p-value was $p < 0.05$. The p-values are given as follows: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$. Data are presented as mean ± SEM.

4. Results

Single mEHT treatment didn't affect the growth of TNBC tumors, however 3 × or more repeated treatments resulted in significant reduction of tumor growth. Five × mEHT treatment was able to reverse tumor growth and turn the growth curve into tumor-size reduction. The final volume of the tumors, measured by caliper and ultrasound

demonstrated also significantly smaller tumors after 3 × or 5 × mEHT, which was corroborated by the tumor weights. The repeated treatments however didn't have any observed toxic effect on the mice, as their body weight didn't change significantly, compared to sham controls. Histological evaluation of H&E stained samples showed an increase in tumor tissue destruction after 3 × and 5 × treatments, however in the long term treatment, sham tumors also developed spontaneously damaged areas. Cleaved caspase-3 immunostaining revealed, that the main mechanism of tumor tissue destruction was caspase-related apoptosis, as the cC3+ areas highly overlapped with the destructed tumor areas seen on H&E. Caspase-independent apoptosis marker, AIF showed no difference at any time point after treatment. Around the damaged area, significant Hsp70 expression was observed, which could be interpreted as a protective mechanism of the still living, injured cells. As a part of the multiplex assessment, we performed Next Generation Sequencing from the RNA (RNA Seq), isolated from the tumor tissue (RNA Seq), 24 hours after the last mEHT treatment. The NGS analysis identified 290 Differentially expressed (DE) genes, and revealed, that the majority of the DE genes were upregulated. A pathway analysis with the GO database demonstrated, that the most upregulated DE genes were part of the Response to stimulus pathway (GO:0050896). This pathway contains several types of genes, classically interpreted as part of the Acute Phase Reaction: complement factors, protease

inhibitors, haptoglobin, coagulation factors. Screening of the NGS data revealed, that 13 Acute Phase Protein genes in total were present as upregulated DE genes after mEHT treatment: Itih2, Itih4, Serpina3n, Serpina3c, Serpina3m, Fgb, Fgg, Hp, Ptx3, Cfd, C4b, Hc, C1s1. NGS results were confirmed by Nanostring analysis withcustom gene panel of 134 genes. Absolute RNA count data confirmed, that 77,6 % of the custom gene panel and all of the expression change directions were the same as seen on the NGS. To investigate protein level changes, a mass spectrometry (MS) analysis was performed. 69.2 % of the APPs were identified successfully at the protein level and 88.9 % of these proteins were significantly upregulated. The three multiplex methods give a strong and complemental evidence about the presence of a local Acute Phase Response induced by the mEHT treatment as an extracellular defense mechanism of the tumor. Two factors with the highest absolute RNA count, C4b and Hp were further analysed. Time kinetic experiment revealed that both C4b and Hp expression reached a peak upregulation 24 hours after mEHT treatment. Hp expression decreased back to sham level, while C4b expression stayed high even 72 hours after the last mEHT. To determine the source of the APPs, 4T1 cells in vitro monoculture were treated with mEHT, then C4b and Hp expressions were measured. qPCR data corroborated, that the source of the C4b and Hp were the cancer cells. Moreover, in KRIBB11 (a heat shock inhibitor) treated tumors the mEHT-induced C4b but not Hp

expression was inhibited. Both C4b and Hp expression correlated with Hsp70 expression.

Our data suggests, that in addition to the intracellular protective response (heat shock response), cancer cells may develop an extracellular defensive mechanism, with the production of APPs (complement factors, protease inhibitors, fibrinogens, haptoglobin) upon treatment with mEHT. The two machinery may cooperate to protect the cancer cells from therapy-induced damage. Those factors, that show the most robust upregulation can serve as therapeutic targets. Inhibition of these factors and mechanisms may enhance the therapeutic effect of mEHT and probably other, heat shock or local APP production inducing treatment modalities (such as chemo- or radiotherapy).

5. Conclusions

This study synthesizes the tumor-growth inhibitory effects and multiplex analysis data about the pathways activated by modulated electro-hyperthermia in mouse TNBC model.

Novel observations of the thesis:

- Repeated modulated electro hyperthermia (mEHT) inhibited tumor growth in the 4T1/4T07 TNBC mouse isografts. Tumor size reduction become manifest after > 3 treatments in our model.

- mEHT treatment induced a local acute phase protein (APP) production in the still-living cancer cells.
- Protease inhibitors, haptogobin, complement and coagulation factors demonstrated the most significant response which are all considered acute phase proteins. This local APP production may serve as a mechanism to protect cancer cells, that received a non-lethal dose of mEHT and did not comitt to cell death.
- 4T1 cancer cells express C4b and Hp as a response to mEHT treatment at the mRNA level in 4T1 monoculture in vitro.
- Local Hp and C4b, induced by mEHT was most significant 24 hours after last treatment. Hp production returned to baseline, while C4b production stayed high even 72 hours after the last mEHT treatment.
- The inhibition of the heat-shock response by KRIBB11 was accompanied by inhibition of mEHT-induced C4b upregulation in vitro.
- Hsp70 expression correlated with C4b and Hp expression 2 hours after treatment, in vitro.

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