VASCULAR AND HYPOXIA DYNAMICS OF MURINE TRIPLE-NEGATIVE BREAST CANCER IN RESPONSE TO MODULATED ELECTROHYPERTHERMIA

PhD thesis booklet

Syeda Mahak Zahra Bokhari

Semmelweis University Doctoral School
Theoretical and Translational Medicine Division

Supervisor: Péter Hamar, MD, PhD, D.Sc
Official reviewers: Éva Szőke, PhD
Dániel Sándor Veres, PhD

Head of the Complex Examination Committee: György Reusz, PhD, D.Sc

Members of the Complex Examination Committee:
Tamás Radovits, PhD
Charaf Hassan, PhD, D.Sc

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1. Introduction

Triple-negative breast cancer (TNBC) is an aggressive breast cancer subtype, characterized by a lack of expression of progesterone- (PR), estrogen- (ER), and human growth factor- receptor 2 (HER-2). TNBC accounts for 15-20% of all breast cancer types. TNBC is a highly aggressive cancer with high metastatic potential and recurrence rates. Current treatment modalities include chemotherapies however there is no standard/specific treatment available. New therapeutic modalities, such as (neo)adjuvant therapies may help to manage TNBC.

Modulated electrohyperthermia (mEHT) is an efficient non-invasive adjuvant treatment with prominent clinical benefits. mEHT provides cytotoxic effects by loco-regional targeting of tumor tissue by amplitude modulated (AM), 13.56 MHz frequency (radiofrequency) electromagnetic field. Mechanistically, mEHT benefits from the altered cell membrane profile and metabolism of tumor cells. Tumor cells, owing to their altered bioelectric properties, absorb more energy than healthy surrounding cells, enabling mEHT to induce specific tumor damage and cell death.

Tumor vasculature is a complex factor in tumor cell survival and proliferation. The abnormal morphology of tumor vasculature hinders blood perfusion and hence reduces the oxygen supply in the tumor core, promoting a hypoxic tumor microenvironment. Tumor vascularization and oxygenation response to hyperthermia are variable and complex. Effects of HT on blood flow and vascular damage have been extensively researched. However, the effect of mEHT on blood flow and vascular damage still needs investigation. In clinical settings, patients receive multiple mEHT treatments. i.e. an average of 28-30

sessions during treatment. It is, therefore, important to investigate the effect of repeated mEHT on the vascular and hypoxic dynamics of TNBC. Furthermore, it is interesting to investigate if mEHT potentiates anti-cancer effects of anti-cancer drugs as an effective adjuvant therapy.

2. Objectives

Studies described in the present dissertation are aimed at investigating the vascular and hypoxic response of triple negative breast cancer in response to modulated electrohyperthermia and efficacy of mEHT as an adjuvant therapy potentiating anticancer drug activity.

- To investigate the effect of repeated modulated electrohyperthermia on the vasculature of TNBC tumors.
- The time kinetic assessment of vascular and hypoxic dynamics.
- To investigate the role of mEHT in initiating angiogenesis.
- Inhibition of mEHT induced hypoxia and angiogenic repair by repurposing digoxin as an inhibitor if HIF1-α transcription.
- To investigate the effect of mEHT therapies combined with either digoxin and/or SC-23

3. Methods

3.1 Cell culture

4T07 and 4T1 cells were grown as adherent culture in Dulbecco's Modified Essential Medium (DMEM 4.5 g/L glucose with L-glutamine, #12-604F, Lonza A. G., Basel, Switzerland) supplemented with 10% Fetal Bovine Serum

(FBS Catologue#ECS0180L, Euroclone S.p.A., Pero, Italy), and 10% Penicillin-Streptomycin (#17-602E, Lonza A. G., Basel, Switzerland).

3.2 In-vivo model

Six-to-eight-week-old female BALB/c mice were raised in the SPF animal facility of the Department of Oncology, Semmelweis University with ad libitum access to standard rodent chow and water, under 12 h dark/12 h light cycles. Animals were anesthetized for tumor cell-inoculation with isoflurane (Baxter International Inc., Deerfield, IL, USA) in 4–5% concentration for induction and 1.5–2% to maintain anaesthesia with 0.4–0.6 l/min compressed airflow. 1 × 106 4T1 cells in 50ul Phosphate Buffered Saline ((PBS) without Calcium and Magnesium #17-516F, Lonza A.G., Basel, Switzerland) solution were subcutaneously inoculated by 50μL Hamilton syringe (Hamilton Company, Reno, NV, USA). Inoculation was made orthotopically into the 4th mammary gland's fat pad in each mouse. Eight days after inoculation, tumors were measured with digital calliper and ultrasound and mice were randomized into mEHT and sham-treated groups according to tumor size and body weight. Mice were injected daily with 2mg/kg dose of digoxin (Merck Life Science Kft. D6003) or saline for eight days. Twenty-four hours after the last treatment mice were euthanized by cervical dislocation under anaesthesia. The tumors were resected and cleaned of the surrounding connective tissue, fat, and skin. The condition of the internal organs (bowels, urinary bladder, spleen) and possible adherences between the tumor and muscles were inspected. Tumors were cut

in half along their longest diameter, one half was placed in a 4% buffered formaldehyde solution (Molar Chemicals Kft., Halásztelek, Hungary). The other half of the tumors were frozen in liquid nitrogen for molecular analysis (RNA isolation, RT-PCR). For time kinetics experiments, tumors were harvested at different time points: 4, 12, 24, 48, and 72 h after the last treatment and mice were injected with pimonidazole hydrochloride (HypoxyprobeTM1, HPI catalogue no. HP1-200) by tail vain injection an hour before tumor harvest. Interventions and housing of the animals conform to the Hungarian Laws No. XXVIII/1998 and LXVII/2002 about the protection and welfare of animals, and the directives of the European Union. Animal experimental protocol was approved by the National Scientific Ethical Committee on Animal Experimentation under Nos. PE/EA/633-5/2018 and PE/EA/50-2/2019.

3.3 In-vivo mEHT treatments

Tumors were treated 3–5 times with the newly developed rodent modulated electro hyperthermia device as described previously 52, 53. The principle of the treatment is a capacitive coupled, amplitude-modulated, 13.56 MHz electromagnetic field which transfers energy to the tumors. Animals were placed on a heating pad (in vivo applicator), functioning as the lower electrode, and connected to the LabEHY modulated electro hyperthermia 200 device with heating and radiofrequency (RF) cable. The abdominal area below the mobile electrode and the back of the mice was shaved before the treatments to enable electric coupling. Treatments were performed with a LabEHY 200 device in a temperature-driven way, for 30

min with 0.7 ± 0.3 watts after a 5-min-long warmup. Temperature monitoring was performed with optical temperature sensor Luxtron (Oncotherm Ltd., Budaörs, Hungary). Temperature parameters were set and monitored as per our previously demonstrated guidelines 53. During sham treatments, the electromagnetic field was turned off but all other conditions (heat pad temperature, upper electrode position) were similar to the mEHT treatment.

3.4 Matrigel plug assay and flow cytometry

To visualize and analyze the effect of mEHT on vascularization in vivo we performed a Matrigel plug assay. Eight to ten-week-old male C57BL/6 mice were raised in the Department of the Animal Facility of the Basic Medical Science Center of Semmelweis University. 500μL liquid Matrigel (BD Biosciences) containing 600 ng/ml bFGF were injected subcutaneously into the left and right groin regions of mice. On day 3 the right-side plugs were treated with mEHT and the left-side plugs were used as controls. The treatment was repeated twice every other day. After 8 days the plugs were excised and subjected to measure the hemoglobin content by hemoglobin Assay Kit (Sigma-Aldrich,) or to flow cytometry.

Matrigel plugs were treated with Liberase TM (Roche Diagnostics) at 37 C for 30 min. The digested plugs were then filtered through a 70-um cell strainer and red blood cells were eliminated by Red Blood Cell Lysis buffer (BioLegend; San Diego, CA, USA), centrifuged for 5min at 350xg, washed with PBS, and fixed and permeabilized

with Intracellular Fixation & Permeabilization Set (eBioscience). TruStain FcX antibody (Biolegend; San Diego, CA, USA) was used for blocking the non-specific binding of IgG to the Fc receptors. Immunostaining was performed by incubating the cells with monoclonal antibodies for 30 min on ice. The following antibodies were used: PE anti-mouse CD31 Antibody and APC anti-mouse CD45 Antibody (Biolegend) San Diego, CA, USA). Flow cytometry was performed with a FACS Calibur (Becton Dickinson, Mountain View, CA, USA). Frequency and intensity measurements were calculated in CellQuest software (Beckton Dickinson).

Table 1. Antibodies used for Flow cytometry.

Antibody	***Type***	***Catalogue no.***	***Vendor***
APC CD-45	Mouse, mAb	103111	Biolegend
CD-31	Mouse, mAb	102407	Biolegend

3.5 Histology and Immunohistochemistry

Formalin-fixed tumor samples were dehydrated and embedded in paraffin. Serial sections (2.5 µm) were cut and mounted on salinized glass slides, and kept in a thermostat at 65 °C for 1 h. Sections were dewaxed and rehydrated for hematoxylin-eosin (H&E) staining and immunohistochemistry (IHC). Endogenous peroxidases were blocked for 15 min using 3% H2O2 in methanol. For antigen retrieval, slides were subjected to constant heating for 20 min in (Tris-EDTA (TE) buffer pH 9.0 (0.1 M Tris base and 0.01 M EDTA) or (citrate buffer pH 6.0 (Dako,

Glostrup, Denmark)) for CD-31 and anti-pimonidazole staining respectively, using an Avair electric pressure cooker (ELLA 6 LUX(D6K2A), Bitalon Kft, Pécs, Hungary). Followed by a 20-min cooling with an open lid. The non-specific proteins were blocked by incubation with 3% bovine serum albumin (BSA, #82-100-6, Millipore, Kankakee, Illinois, USA) diluted in 0.1 M Tris-buffered saline (TBS, pH7.4) containing 0.01% sodium azide for 20 mins. The sections were incubated with the primary antibodies diluted in 1% BSA/TBS + TWEEN (TBST, pH 7.4) (Table 1.) overnight in a humidity chamber. Peroxidase-conjugated anti-rabbit & anti-mouse IgGs (HISTOLS-MR-T, micropolymer -30011.500T, Histopathology Ltd., Pécs, Hungary) were used for 40 min incubations and the enzyme activity was revealed in 3, 3'-diaminobenzidine (DAB) chromogen/hydrogen peroxide kit (DAB Quanto-TA-060-QHDX-Thermo Fischer Scientific, Waltham, MA, USA) under microscopic control. All incubations were at room temperature with the samples washed between incubations in TBST buffer for 3 × 3 min. Digital evaluation of Tumor Destruction Ratio (TDR%) on H&E slides and the CD31 and pimonidazole staining was performed using the case viewer software as described earlier 53. Red blood cell covered area was quantified by the CaseViewer software, RBC covered area was masked relative to the whole annotated tumor area by double masking. Number of viable blood capillaries per tumor area was manually counted, viable vessels were characterised as circular, luminal, surrounded by living

cells, visibly containing RBCs with area no less than 100um2.

Table 2. Antibodies used for Immunohistochemistry.

Antibody	***Type***	***Catalogue no.***	***Dilution***	***Vendor***
Anti-Pimonidazole	Mouse, mAb	4.3.11.3	1:50	Hypoxyprobe™ M
CD-31	Rabbit, mAb	77699S	1:100	Cell Signaling

3.6 Western Blot analysis for HIF-1α

Total protein isolation was performed with TRI reagent (Molecular Research Center lnc., Ohio, USA) according to the manufacturer's instructions. 20µg protein was loaded per well and fractionated on 12% SDS-PAGE gel and transferred to PVDF membrane. Membrane was cut to two in order to probe the same membrane for two proteins simultaneously. Membranes were probed with primary antibody specific for HIF-1α and β-actin overnight at 4°C. Membrane was then incubated with HRP conjugated secondary antibody for an hour. Chemiluminescent signal was detected by SuperSignal™ West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific, catalogue # 34578). Chemiluminescent signal was detected by X-ray film and blot was analysed by ImageJ software.

Table 3. Antibodies used for Western Blot.

Antibody	***Type***	***Catalogue no.***	***Dilution***	***Vendor***
HIF-1α	Mouse, mAb	sc-13515	1:200	Santa Cruz Inc.
β-actin	Mouse, mAb	ab6276	1:5000	Abcam
Anti-mouse IgG	-	7076	1:3000	Cell signaling

3.7 Statistical analysis

Statistical analysis was done using The GraphPad Prism software (v.6.01; GraphPad Software, Inc., La Jolla, CA, USA). Unpaired Mann-Whitney nonparametric tests were performed in the comparison of sham and mEHT treated groups. Follow-up examinations for more than two groups were statistically evaluated with two-way ANOVA or one-way ANOVA with Tukey correction. Differences were considered statistically significant as *$p < 0.05$, **$p<0.01$, ***$p<0.001$. Data are presented as mean ± SEM.

4. Conclusion

The studies mentioned in this dissertation investigated the molecular basis of stress responses initiated in response to modulated electrohyperthermia in triple negative breast cancer. The main findings demonstrated that:

- Repeated modulated electrohyperthermia (mEHT) induced vascular damage in triple negative breast cancer models.
- mEHT mediated vascular damage triggers hypoxia in a time dependant manner.
- mEHT promotes proliferation of $CD31^+$ endothelial cells in non-cancerous matrigel implants.

- An angiogenic repair is initiated in response to mEHT in a time related manner.
- Digoxin can be repurposed as an anticancer drug as it reduces tumor proliferation and HIF1-α expression.
- Combining mEHT with anti-cancer drugs (digoxin, SC-236) slows tumor proliferation in a short period of time as compared to monotherapy at the same dosage.
- Combination therapies mEHT+digoxin and mEHT+ SC-236 had synergistic anti-tumor effects.

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