Molecular mechanisms of modulated electrohyperthermia (mEHT) induced tumor damage

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Molecular mechanisms of modulated electrohyperthermia (mEHT) induced tumor damage

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Abstract
Modulated electro-hyperthermia (mEHT), a non-invasive, loco-regional complementary of radio- or chemotherapy, can by itself induce selective heat shock and cell stress in malignant tumors at ~42oC. Based on the published results we briefly summarize what has been revealed on the molecular background of tumor damage caused by mEHT treatment.

A single mEHT shot of 30-60 min provoked significant upregulation of γ-H2Ax (indicating DNA double strand brakes) and tumor destruction in colorectal cancer models, both in vitro and in vivo, dominantly following programmed tumor cell death mechanisms. Apoptotic response was diverse based on the (epi) genetic makeup of treated tumors and following both extrinsic (casp-8+) and intrinsic (translocated Bax & Cytochrome C) caspase-dependent (casp-3+; in C26), or AIF-mediated (in p53 mutant HT29) caspase-independent pathways. Treatment response in C26 in vitro involved the upregulation of Ser15 phospho-p53 (indicating escape from Mdm2 control) and p21waf1 (the mediator of cell senescence), accompanied by the elevation of the pro-apoptotic PUMA, Bax and Bak-1 and the downregulation of the antiapoptotic XIAP, Bcl-2 and Bclx. Furthermore, mEHT treatment synergized with Doxorubicine chemotherapy. In histiocytic lymphoma (U937) both extrinsic and intrinsic caspase-dependent apoptosis was driven by phosphorylation of the c-Jun N-terminal kinases (JNK).

In vivo, early apoptosis was supplemented by complete cell cycle arrest shown by Ki67 negativity, and the occurrence and release of DAMP (damage associated molecular pattern) signals including chaperons such as calreticulin, Hsp70 and Hsp90 and the high mobility group1 (HMGB1) protein. After single treatment, the progressive tumor damage and accumulation of CD3 positive T-cells, including granzyme B+/CD8+ cytotoxic cells (granzyme B+/CD8- NK cells) as well as S100+ antigen presenting dendritic cells (APC), were consistent with a secondary, immunogenic cell death (ICD) mechanism added to the primary effect of mEHT. Furthermore, treatment response could be associated with elevated levels of glycolytic enzymes in vivo, and with increased lactate production and reduced buffer capacity (and pH) in cultures. mEHT treatment also supported antitumor immune response when combined with tumor-specific, intratumoral dendritic cell delivery involving tumor sites distant from the treated focuses (Abscopal effect).

In summary, radio- or chemotherapy can be supported by the inherent antitumor effects of mEHT, which can induce diverse, tumor-specific apoptosis pathways and antitumor immune response too. Besides direct heat induction in the extracellular space due to elevated glycolysis (Warburg-effect) and ion-concentration in cancer, mEHT may also act directly on cell membrane rafts (where local electric loss/absorption peaks), which concentrate ion
channels and transmembrane receptors. These features may explain the higher efficiency of mEHT compared to traditional hyperthermia under the same temperature.

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ICH5 Congress, Budapest
September 28-29, 2018

mEHT of 13.65 MHz – selective tumor targeting

Enrichment of electric field in malignant tumors

- Elevated: glucose uptake, aeroe glycolysis (Warburg-effect)
  lactate H+ & other ion concentration & permittivity

- Dielectric polarization/rotational friction – heat
mEHT of 13.65 MHz – Significant tumor destruction

- Mechanism: Programmed tumor cell death

C26 mouse CRC

\( \text{in vivo} \)

Untreated

Treated

Synergy
Heat stress +
Direct effect of EF

Additional effects compared to conventional heating


mEHT effects: in vivo & in vitro tumor models

Mouse: allo-, xenografts

Temperature control

Tumor cell cultures

Temporal tumors
- right leg - mEHT treated
- left leg - control

mEHT: single/repeated 30 or 60 min
42 ±0.5°C (Lab EHY 100)

mEHT: mono-, or combined with chemo- or radio- or DC therapy

Published results from:
- Yonsei University College of Medicine Seoul, South Korea
- National University, Seoul, South Korea
- Tottori University, Japan
- Chiba University, Japan
- Toyama University, Japan
- Memorial Hospital, Taipei, Taiwan, ROC
- Chung Yuan Christian University, Taoyuan City, Taiwan, ROC
- Semmelweis University, Budapest, Hungary

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mEHT induced programmed cell death (42°C)

Colorectal cancer (CRC) cell lines: HT29 human (TP53 mutant); C26 mouse

APOPTOSIS

DNA fragmentation

Early heat shock/cell stress & apoptosis response

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mEHT induced death receptor mediated extrinsic pathway

**hHT29 (TP53 mutant) CRC xenograft**

**TRAIL-R2** (Death Receptor 5)

<table>
<thead>
<tr>
<th>mRNA (%)</th>
<th>8 h</th>
<th>14 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated</td>
<td><strong>3</strong></td>
<td><strong>2</strong></td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

**mRNA levels**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Ratio to GAPDH reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAIL-R2 (DR5)</td>
<td><strong>3.5</strong></td>
</tr>
<tr>
<td>FAS</td>
<td>2.5</td>
</tr>
<tr>
<td>FADD</td>
<td>2</td>
</tr>
</tbody>
</table>

**Targeted therapy:** rh-Apo2L/TRAIL and MAbs (HGS ETR2/lexatumumab) - agonist

- Support cytotoxic chemo- and radiation therapy in breast cancer & CRC
- Phase-I-II trials have been running

**mEHT induced Caspase independent & dependent apoptosis**

**mHT29 CRC** (mTP53)

**AIF (apoptosis inducing factor) translocation** (24h)

**mC26 CRC** (wTP53)

**Extrinsic Caspase dependent**

Cleaved-Caspase 8

Cleaved-Caspase 3

24h post-treatment
mEHT induced Caspase-dependent intrinsic pathway

**Bax - mitochondrial translocation**

Untreated | Treated
--- | ---
14h | 14h

**Cytochrome C - cytoplasmic release**

Untreated | Treated
--- | ---
14h | 14h

**mEHT-induced DNA double strand brakes**

H2Axy - DAPI | Ki67 - DAPI | Merged
--- | --- | ---

H2Axy - DAPI | p21\textsuperscript{waf1} - DAPI | Merged
mEHT-induced DNA damage - apoptosis

H2Axγ c-Caspase 3 DAPI

mEHT induced apoptosis and cell cycle arrest

mC26 CRC

Ki67

Proliferation rate > 90%

72h

mEHT - Complete cell cycle arrest

24h

n=3

* * *

Nuclear phospho-p63 (SER15)

mEHT

control

p21 mRNA mRNA levels

Relative mRNA expression

Cont 1 Hr 3 Hr 9 Hr 24 Hr

24h

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Spatiotemporal „danger” signaling – systemic effect

Damage associated molecular patterns (DAMP)

- ATP „find me” signal
- Calreticulin (CRT) „eat me” signal
- HMGB1 „danger” signal
- HSP70
  - Granzyme B
  - endocytosis

• DC maturation, activation
• Tumor antigen processing
• T-cell & NK-cell activation

• Antracyclins
  - Doxorubicin
• UV or γ-irradiation
• EGFR
  - immunotherapy
• Capsaicin

- Antitumor immune response
- Immunogenic cell death (ICD)

C26 CRC allograft

Calreticulin membrane translocation


mEHT mC26: CRT in vitro

Hsp70 membrane translocation

C26 CRC allograft

Hsp70 WGA DAPI

untreated

HSP70

45h

Relative Hsp70 positive cell number per 40x FOV (%)

untreated

mEHT sham

48h

HMG1 release – cytoplasmic & extracellular

HMGB1 DAPI

untreated

untreated

Treatment

48h

HMGB1 positive area (µm²)

untreated

mEHT sham
mEHT combined with MTE (T-cell promoter)

**Systemic (abscopal) effect**

Tumor Destruction Ratio

\[ \text{TDR} = \frac{\text{Damaged area}}{\text{Whole area}} \]

**In mEHT treated & mEHT+MTE treated & in mEHT+MTE treated opposite site**

<table>
<thead>
<tr>
<th></th>
<th>24 h TDR (% of sham)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mEHT</td>
<td></td>
</tr>
<tr>
<td>mEHT+MTE</td>
<td></td>
</tr>
<tr>
<td>MTE</td>
<td></td>
</tr>
<tr>
<td>sham</td>
<td></td>
</tr>
</tbody>
</table>

**Calreticulin**

12h

Overlapping CRT & WGA (%)

<table>
<thead>
<tr>
<th></th>
<th>untreated</th>
<th>treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>mEHT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mEHT+MTE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sham</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Hsp70**

48h

Relative Hsp70 positive cell number per 40x FOV (%)

<table>
<thead>
<tr>
<th></th>
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<th>treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>mEHT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mEHT+MTE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sham</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Antitumor immune response - local and systemic**

C26 CRC allograft

Elevated number of antigen presenting DC (APC)

S100

48h

<table>
<thead>
<tr>
<th></th>
<th>untreated</th>
<th>treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>mEHT</td>
<td></td>
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<tr>
<td>mEHT+MTE</td>
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<td></td>
</tr>
<tr>
<td>sham</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Massive T-cell infiltration

CD3

72h

<table>
<thead>
<tr>
<th></th>
<th>untreated</th>
<th>treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>mEHT</td>
<td></td>
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<tr>
<td>sham</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Antitumor immune response – immunogenic cell death

48 h post-mEHT

T-cell infiltration, negligible regulatory T-cells

C26 CRC allograft

1

Single mEHT shot
Progressive
• accumulation of immune cells &
• tumor damage

Immunogenic cell death
ICD

Cytotoxic T-cells & NK cells + Macrophages

Combination of mEHT + DC therapy

mC26 in vitro & allografts

Enhanced immune response
Tsang et al. (Taiwan) BMC Cancer, 2015, 15:708

Elevated Hsp70 release in vitro

(a) CT26
(b) HSP70 release

HSP70
HMGB1
β-actin

37°C 42°C mEHT

Tumor antigen+Hsp70 activated DC

Elevated T-cell activation (& cytotoxicity)

Reduced tumor volume

No tumor after rechallenge

Control DC mEHT mEHT+DC

30 days after 1st challenge

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mEHT + DC therapy – systemic „abscopal” effect

Reduced tumor sizes distant from the mEHT treatment site

Quin et al. (Japan) Oncology Reports 2014, 32:2373-2379.

- Elevated CD3+ and CD8+ T-cells & S100+ antigen presenting DCs
- Reduced FoxP3+ regulatory T-cells

GP96/Hsp90B1 mRNA upregulation in tumor tissues

mEHT promoted radiation damage by inhibiting HIF1α

FSall allograft (fibrosarcoma)

Kim et al. (Korea) Int J Hyperthermia. 2018, 34:276-283.

Oncothermia Journal, Volume 24, October 2018
Combination of 2x30’ mEHT + Doxorubicin

**mC26 CRC in vitro**

**Cell viability: Resazurin assay**

- Control
- mEHT
- Dox (1 μM)
- mEHT+Dox (1 μM)

Viability (% of control)

- 24 h
- 48 h

n=3

**Cell loss**

- Control
- mEHT
- Dox
- mEHT+Dox

Number of cells (% of Control)

n=5

24h

**mEHT - dominantly apoptosis**
**Doxo - dominantly necrosis**

Additive effect in reducing viability & enhancing cell death by the combination therapy

Comparison of mEHT with conventional HT or cRF therapy

**hU937 histiocytic lymphoma**

Upregulation of Fas, Casp-8 and Casp-3 & phosphorylation of JNK

- Mitochondrial membrane potential
- Intracellular Ca²⁺

Treatment: 42°C

**hHepG2 HCC**

Upregulation of ROS, ex.Hsp70 & CRT; Casp-8 and Casp-3

- ROS levels
- Extracellular Hsp70
- Cell membrane Calreticulin

**mRNA expression array**

- p-JNK → p-p53 (SER6)

44°C

CTRL  WHT  mEHT

54 kDa  46 kDa
mEHT upregulated Septin-4 promoted p53 functions

HepG2 & HuH7 HCC in vitro & in vivo xenograft

Transcriptomic analysis of gene expression by RNA sequencing

Upregulation of Septin-4

SEPT4 gene, encodes the inhibitor of apoptosis proteins (IAP) antagonist ARTS

Sept4/ARTS is required for stem cell apoptosis and tumor suppression.

Upregulation of p53

Reduced colony formation

Reduced HepG2 tumor size

Molecular pathways involved in EHT effects

PRIMARY effect
mEHT induced heath/cell stress

Upregulation, translocation and release of DAMPS

SECONDARY effect
Support of antitumor immune response (ICD)

Oncogenic Survival signals

Caspase independent apoptosis
- AIF (TP53 mutant)
- Granzyme B (Hsp70)

Combinations additive effect:
- Dox
- DC
- radiotherapy
Common features of mEHT & Prediction

**COMMON**
- The extrinsic apoptosis pathway was involved: cell membrane effect
- P53<sup>wt</sup> activation was frequent: caspase-dependent apoptosis + senescence

**DIFFERENT**
- Extent of tumor damage & the preferred damage signaling pathway(s) are tumor (type) dependent
- & determined by inherent epi-/genetic make up

*(the same molecular events in the endogenously damaged areas in controls)*

**mEHT**

**PREDICTIVE BIOMAKERS**
- Epi-/genetic predisposition
- Oncometabolit levels
- Metabolic enzyme others ???

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