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Supplementary Material

Sub-lethal hyperthermia promotes epithelial-to-mesenchymal-like transition of breast cancer cells: Implication of the synergy between hyperthermia and chemotherapy

Materials and methods

Cancer Cell Preparation

MCF-7 and MDA-MB-231 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium and Dulbecco's Modified Eagle Medium (DMEM), respectively, which were supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin (Life Technologies, Cergy Pontoise, France). The cells were then grown for 48 h in an incubator (Sanyo, MCO18AIC), maintained with 5% CO_2 at 37 °C.

Insufficient Hyperthermia Treatment

Hyperthermal stress was induced by elevating the temperature of the incubator (CO2CELL50, Revodix) to 42 °C, 47 °C, and 52 °C. After 45 min of exposure, cells were immediately moved back into an incubator with temperature set at 37 °C and maintained for 1-24 h. The viability, EMT characteristics, and chemo-sensitivity of the heat-treated cells were investigated and compared with those of the cells without heat exposure.

Transforming Growth Factor Beta-1 (TGF-β1) Treatment

MCF-7 and MDA-MB-231 cells were treated with TGF-β1 (Thermo Scientific, Wilmington, DE, USA), one of the most well-established EMT inducers [R1]. The cells were incubated in pure RPMI or DMEM with 10 ng/mL TGF-β1 for 48 h. Cell morphology and EMT-related characteristics were examined and compared with those of heat-treated cells.

Cell Viability Analysis

The cell viability was measured using two-colored live/dead cell viability assay kit (Life technology), as described elsewhere [R2]. Heat-treated and untreated cells were kept in solution having 4.0 μM of fluorescein isothiocyanate (FITC)-conjugated Calcein acetoxymethyl and Tetramethylrhodamine (TRITC)-conjugated ethidium homodimer, respectively, for 20 min. Cells were then washed with phosphate-buffered saline (PBS) solution. Viability of cancer cells were measured by comparing the number of cells expressing Calcein acetoxymethyl to the total number of cells.

Cell Morphology Analysis

Cells were incubated in 24-well plate for 24 h at a density of 5×10^3 cells per well. Cells were heat-treated as aforementioned and washed five times with phosphate-buffered saline (PBS). Cell images were acquired using phase-contrast microscopy (Eclipse Ti-E, Nikon, Japan) with a 20× objective. In addition to analyzing the morphological changes with phase-contrast images, we also conducted periodic acid-Schiff (PAS) and crystal violet staining to evaluate cell morphologies in more detail. Cells were subsequently examined with light microscopy (Axiovision, Carl Zeiss, Germany) equipped with a camera (Nikon, Japan) with a 10× objective.

In vitro Cell Migration Assay

Migratory properties were examined based on *in vitro* wound healing assay, as described elsewhere [R3]. Briefly, cells were seeded into 6-well plates and grown to 70% confluence. Cells were heat-treated and wounds were created by scraping monolayer of cells with a swab. Cell images were taken after 48 h of incubation and compared with the initial counts.

Immunocytochemistry

Cancer cells were seeded in 8-well culture chamber slides (SPL Life Sciences) at a density of 2×10^4 cells per well and exposed to hyperthermal stress. Cells were then washed with PBS solution and fixed with 4% paraformaldehyde for 15 min. Endogenous peroxidase was blocked by incubating the cells with 1% H_2O_2 in methanol for 30 min at the room temperature. Cells were automatically immune-stained by BenchMark XT Slide Preparation System (Ventana Medical Systems, Inc.). In brief, five primary antibodies, including anti-E cadherin (1:300, DAKO), anti-CK8/18(1:200, DAKO), anti-Vimentin (1:1000, DAKO), anti-Desmin (1:150, DAKO), and anti-p53(1:300, DAKO) antibodies, were applied to quantify the changes in EMT-related properties or demonstrate the cell death mechanism after combined treatment of thermotherapy and chemotherapy. We incubated cells with each of antibodies for an hour and washed with washing buffer (DAKO). Cells were then reacted with EnVisio FLEX/HPR (DAKO) for 40 min and incubated with DAB reagent (3,3-diaminobenzidine tetrahydrochloride, DAKO) for another 10 min. Cells were kept in modified Mayer's hematoxylin solution and stained for 3 min. Positive expression was defined by measuring brown signals in cytoplasm or cell membrane.

Western Blot Analysis

Cells were harvested and lysed with RIPA buffer after the heat treatment. Proteins were quantified using BCA protein assay kit (Pierce), by following the manufacturer's instructions. The supernatants were collected and stored at -80 °C, until they were used for the analysis. The extracted proteins were then mixed with an SDS sample buffer and subjected to 10% SDS-PAGE with running buffer. After the electrophoresis, we used iBlot Dry Blotting System (Thermo Fisher Scientifics) for the protein transfer. The membrane was reacted with iBLot Gel Transfer Stacks polyvinylidene fluoride (PVDF) in TBS-T [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Tween-20 V/V] for 30 min at the room temperature and subsequently incubated with primary and secondary antibodies for 1 h and 2 h, respectively. Anti-heat shock protein 70 (Hsp70, 1:1000, Merck milipore) and Anti-Hsp90 (1:500, Santa Cruz, CA, USA) were diluted in TBS-T and the expression levels of each antibody were analyzed to confirm that increase of Hsps help cells to survive and recover from external stimulus. Expression level of E-Cadherin (1:1000, Merck milipore) and vimentin (1:1000, Merck milipore) were measured and compared with the expression of β-actin (1:1000, Merck milipore), to validate epithelial-to-mesenchymal-like changes after heat treatment. The membrane was then incubated with the secondary anti-rabbit and mouse anti-IgG antibodies (Santacruz) in TBS-T (diluted 1:1000) and immuno-complexes were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce). Finally, blots were developed using FluorChem FC3.

Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

The expression levels of Hsp70 and EMT-related molecules were quantified by qRT-PCR. Total RNA was isolated by Norgen's Total RNA purification kit (Norgen) after 24 h of cell starvation. cDNA was synthesized using TruScrip first strand cDNA synthesis kit (Norgen) according to the

manufacturer's instructions. qRT-PCR was performed using TaqMan Gene Expression Master Mix (Applied Biosystems-Thermo Scientific) and the Light Cycler 480 (Roche). Primer sequences, including HSPA1A (Hs00359163_s1), CDH1 (Hs01023894_m1), VIM (Hs00958111_m1), TWIST1 (Hs01675818_s1), and GADPH (Hs02758991_g1), were purchased from Thermo Fisher Scientific.

Cell Nuclei Analysis

The cell nuclei were examined after DAPI staining. The cells were fixed with 4% paraformaldehyde for 15 min, washed with PBS, and stained with DAPI for 10 min. The cells were washed once again with PBS and analyzed using a fluorescence microscope (Nikon) with a 20× objective.

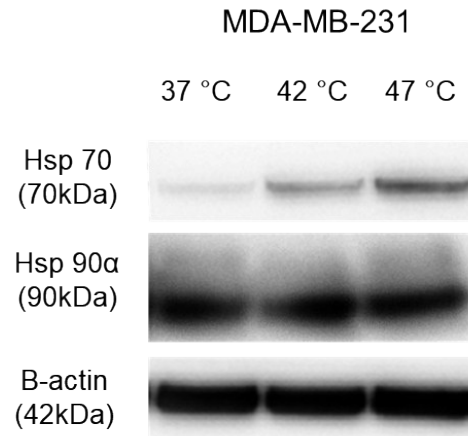

Figure S1. Expression of heat shock proteins after insufficient heat treatment.

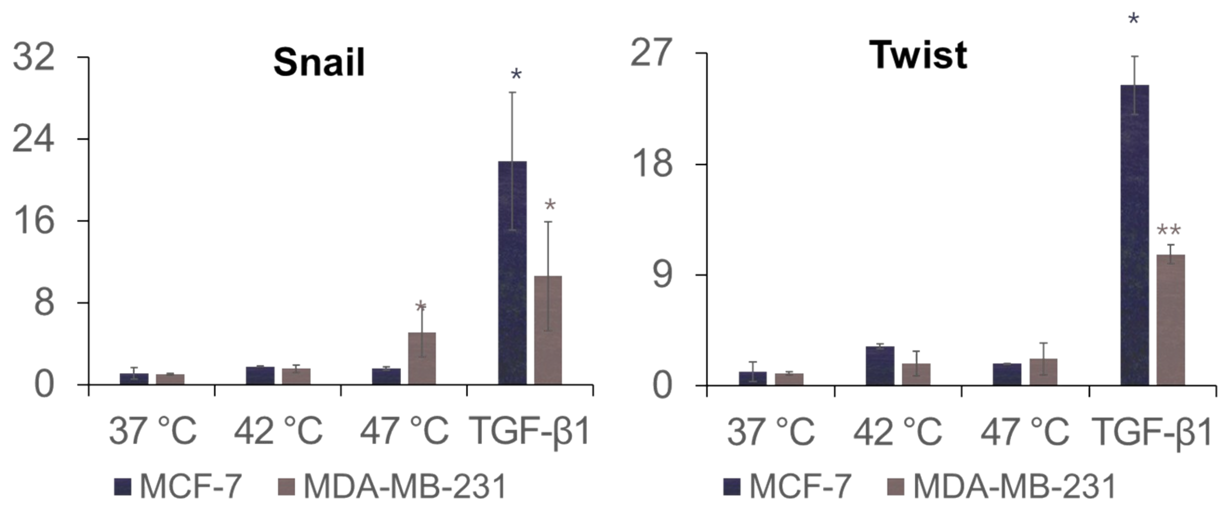

Figure S2. The effect of insufficient hyperthermia on EMT-activating factors.

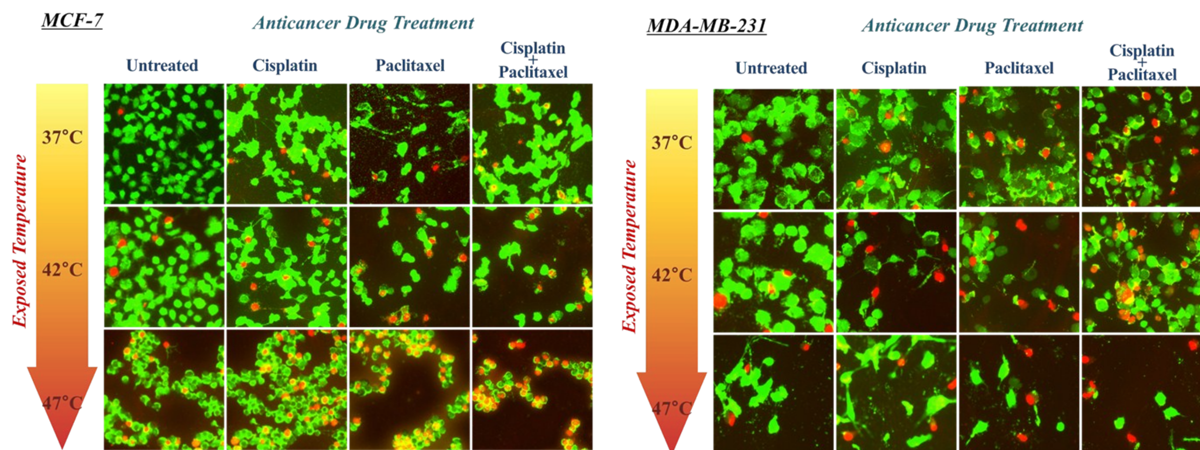

Figure S3. The cell viability after thermo- and/or chemotherapy. Images were taken after staining the cells with live/dead two-colored fluorescence assay.

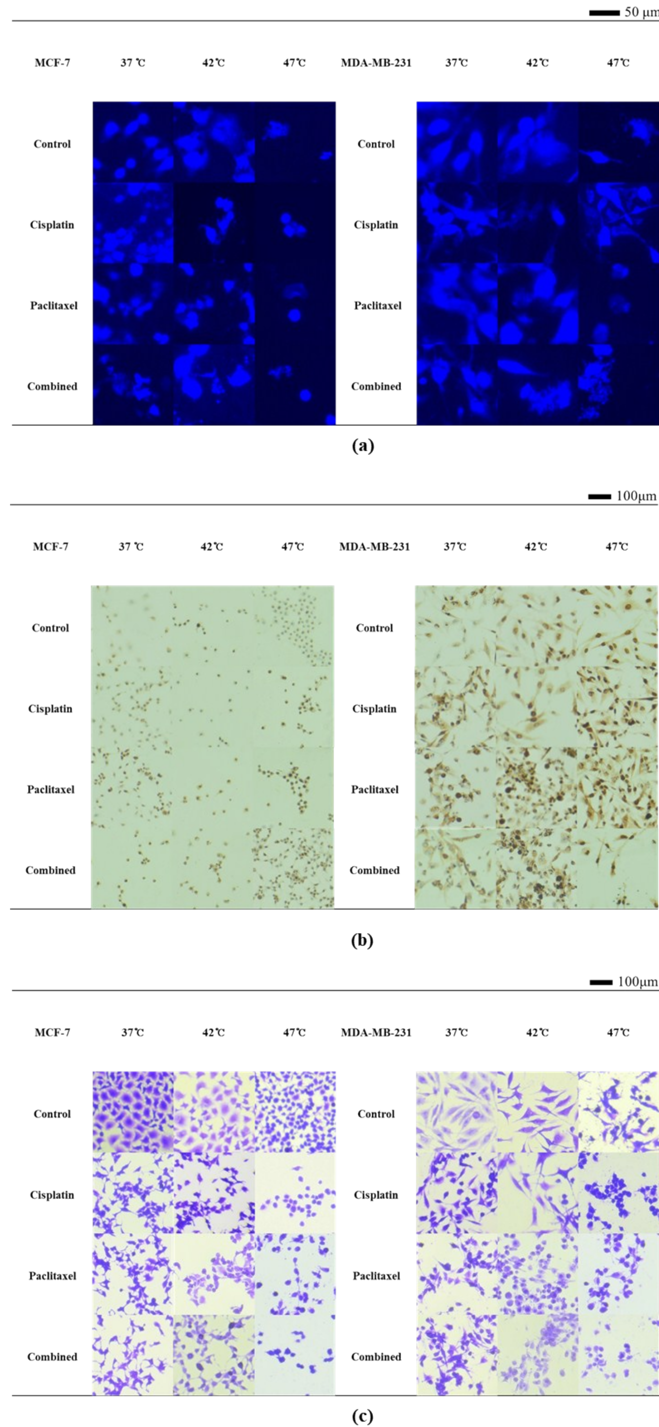

Figure S4. Induction of apoptotic cell death due to cisplatin, paclitaxel, or combined treatment on MCF-7 and MDA-MB-231 cells; (a) cells stained with DAPI after anti-cancer treatment. The stained apoptotic body was observed under a fluorescent microscope using a blue filter; (b) immunocytochemical expression of p53 and (c) crystal violet staining examined under a light microscope.

[R1] J. Bu, Y.-H. Cho, S.-W. Han, Enhancement of isolation sensitivity for the viable heterogeneous circulating tumor cells swelled by hypo-osmotic pressure, RSC Advances, 7 (2017) 49684-49693.

[R2] Y. Kim, J. Bu, Y.-H. Cho, I.T. Son, S.-B. Kang, A viable circulating tumor cell isolation device with high retrieval efficiency using a reversibly deformable membrane barrier, Journal of Micromechanics and Microengineering, 27 (2017) 025015.

[R3] W.-C. Chiu, T.-J. Chiou, A.-N. Chiang, β₂-Glycoprotein I inhibits endothelial cell migration through the nuclear factor κB signalling pathway and endothelial nitric oxide synthase activation, Biochemical Journal, 445 (2012) 125.