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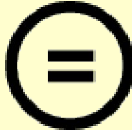

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Electro-hyperthermia inhibits glioma tumorigenicity through the induction of E2F1-mediated apoptosis

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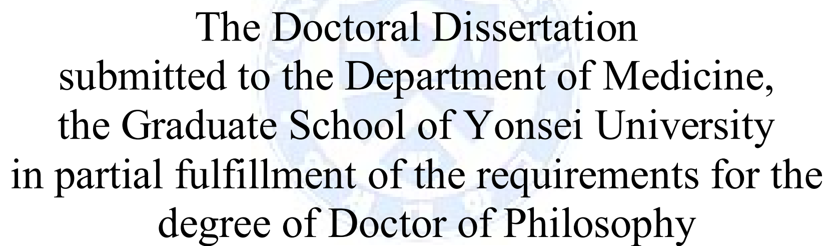
Department of Medicine

The Graduate School, Yonsei University

Electro-hyperthermia inhibits glioma tumorigenicity through the induction of E2F1-mediated apoptosis

Directed by Professor Chang Geol Lee



The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the
degree of Doctor of Philosophy

Jihye Cha

December 2015

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Jihye Cha

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ABSTRACT

Electro-hyperthermia inhibits glioma tumorigenicity through the induction of E2F1-mediated apoptosis

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(Directed by Professor Chang Geol Lee)

Purpose: Modulated electro-hyperthermia (mEHT), also known as oncothermia, shows remarkable treatment efficacies to various types of tumors, including glioma. The aim of the present study was to investigate the molecular mechanism underlying phenotypic changes in oncothermic cancer cells.

Materials and methods: U87-MG and A172 human glioma cells were exposed to mEHT (42℃/60 min) three times with a 2-day interval and subsequently tested for growth inhibition using MTS, FACS and microscopic analysis. To obtain insights into the molecular changes in response to mEHT, global changes in gene expression were examined using RNA sequencing. For *in vivo* evaluation of mEHT, we used U87-MG glioma xenografts grown in nude mice.

Results: mEHT inhibited glioma cell growth through the strong induction of apoptosis. The transcriptomic analysis of differential gene expression under mEHT showed that the anti-proliferative effects were induced through a subset of molecular alterations, including the upregulation of E2F1 and CPSF2 and the downregulation of ADAR and PSAT1. Subsequent Western blotting revealed that mEHT increased the levels of E2F1 and p53 and decreased the level of PARP-1, accelerating apoptotic signaling in glioma cells. mEHT significantly suppressed the growth of human glioma xenografts in nude mice. We also observed that mEHT dramatically reduced the portion of $CD133^+$ glioma stem cell population and suppressed cancer cell migration and sphere formation.

Conclusions: These findings suggest that mEHT suppresses glioma cell proliferation and mobility through the induction of E2F1-mediated apoptosis and might be an effective treatment for eradicating the brain tumors.

Key words: electro-hyperthermia, glioma, E2F1, apoptosis, cancer stem cells

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I. INTRODUCTION

Gliomas are the most common and deadly brain tumors, accounting for ~80% of the primary malignancies of the brain.[1, 2] Despite recently advanced multimodal therapies, such as surgery, chemotherapy and radiotherapy, the overall 5-year survival rate for malignant glioma remains less than 10%.[3] Thus, new approaches to improve the treatment efficacy are needed.

Hyperthermia cancer therapy is currently used either for ablation purposes as an alternative to surgery or in combination with chemotherapy and/or radiation therapy to enhance the effects of those traditional therapies.[4] Modulated electro-hyperthermia (mEHT) is a complementary treatment method effective against various types of tumors, including gliomas.[5, 6] The principle of mEHT is based on the classical hyperthermia, but the difference is

that mEHT is physically designed to promote the direct absorption of the modulated radiofrequency current in the extracellular fluid. In mEHT, the extracellular liquid is heated, creating a slight temperature difference (1/1000℃) between the inner and outer temperature of the cell. As a result, the heat flows into the cell through the membrane until the temperature difference extinct, thereby destroying the membrane of the cancer cells.[7-9] When combined with alkylating chemotherapy or the molecular targeting agent sorafenib, mEHT exhibits synergistic anti-cancer effects in clinical trials with glioma or hepatocellular carcinoma patients.[10, 11] A recent study also showed that mEHT induces an abscopal effect, thereby enhancing the antitumor effects of immune-therapy.[12]

Although the anti-tumor efficacy of mEHT has been extensively studied in both laboratory and clinical settings, the underlying molecular mechanisms of this treatment are not yet clearly understood. In the present study, we aimed to elucidate the molecular mechanisms mediating the phenotypic changes in oncothermic tumor cells using a laboratory mEHT device for *in vitro* experiments and a glioma xenograft model. We also performed RNA sequencing and subsequent protein analysis to understand the mechanism on a whole genome scale. We also performed experiments that detecting $CD133^+$ glioma stem cells, cell migration assay, and sphere forming assay to investigate the effect of mEHT on the cancer stem cells.

II. MATERIALS AND METHODS

1. Cell culture

Human brain cancer cell lines, U87-MG and A172, were purchased from the Korean Cell Line Bank (KCLB, Seoul, South Korea). U87-MG and A172 cells were cultured in MEM and RPMI-1640 medium, respectively, supplemented with 10% of fetal bovine serum (HyClone, South Logan, UT, USA) and 1% of penicillin/streptomycin solution (HyClone), at 37℃ with 5% CO_2. The phenotypes of these cell lines have been authenticated by the KCLB.

2. Modulated electro-hyperthermia (mEHT)

The LAB-EHY100 device (OncoTherm Ltd., Hungary) was used in *in vitro* and *in vivo* mEHT treatment. To mimic clinical conditions, mEHT (3W power, 42℃ for 60 min) was administered to glioma cells three times at 2-day intervals. During the 60 min of mEHT, the radio-frequency (RF) power level was controlled using the fluorotopic temperature measurement system (Luxton m3300; Lumasense, Santa Clara, CA, USA) to maintain the heating temperature at 42℃. For in vivo experiments, four-week old male BALB/c nude mice were purchased from Orientbio (Seongnam, South Korea). To elucidate the efficacy of mEHT against *in vivo* tumors, 1×10^7 U87-MG cells were injected into the right flanks of nude mice and the xenografts were treated with mEHT using the same instrumental conditions on 9, 11, 13, 15,

17, 19 and 21 days after tumor cell injection. The tumor size was measured using a vernier caliper and calculated as (the minor axis^2 × the major axis × 1/2).

3. Measurement of cell proliferation and apoptotic cell death

The cell growth was measured using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. The cells were treated as described above and incubated for 2 hours at 37℃ in a humidified, 5% CO_2 atmosphere. The colored formazan products were determined by measuring the absorbance at 490 nm using a VersaMax microplate reader (Molecular Device, Sunnyvale, CA, USA). Annexin V and PI dual staining was used to detect apoptotic cells, but the cells were not fixed with 70% ethanol. Apoptotic cells were stained using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, San Jose, CA, USA) according to the manufacturer's instructions. The cell death was measured through FACSVerse flow cytometry (BD Bioscience) and quantified using FlowJo software. The induction of apoptosis was also measured using the ApoStrand ELISA Apoptosis Detection Kit (Biomol International, Plymouth Meeting, PA, USA). This method detects denatured single-stranded DNA in apoptotic cells, but not in the necrotic cells or cells with DNA breaks.

4. Clonogenic assay

The effect of mEHT on the clonogenic survival of tumors was studied. The cells treated with mEHT were seeded onto 24-well culture plates at 1×10^3 cells per well. The cells were maintained in culture for 12 days without medium change to let the viable cells propagate to sizable colonies for quantification. The colonies were fixed with methanol-acetic acid 3:1 ratio and subsequently stained with 0.5% crystal violet for 60 min at room temperature. The numbers of colonies containing more than 50 cells were counted.

5. RNA sequencing

Total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA, USA). The quantity and quality of the total RNA were evaluated using RNA electropherograms (Bio-Rad Experion, Hercules, CA, USA) and the RNA quality was assessed based on the RNA quality indicator (RQI). The total RNA from each sample with a RQI value of 8.0 or higher was used. The resulting mRNA samples were processed for the sequencing libraries using the Illumina TruSeq Stranded mRNA sample preparation kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocols. RNA sequencing was performed using the Illumina HiSeq 2500 to generate non-directional, paired-end 100-base-pair reads. Quality-filtered reads were mapped to the human reference genome sequences, hg19 (UCSC Genome

Bioinformatics, https://genome.ucsc.edu) using tophat2 (http://ccb.jhu.edu/software/tophat). The relative transcript abundance was estimated by counting the fragments per kilobase of exon model per million mapped sequence reads (FPKM) and differential expressed genes were evaluated using cufflinks package (http://cufflinks.cbcb.umd.edu). The significantly overlapping pathways and Gene Ontology categories with differential expressed genes were analyzed using DAVID (http://david.abcc.ncifcrf.gov).

6. Western blot analysis

Total cell lysates were prepared by lysing the cells in RIPA buffer (Thermo Scientific, Rockford, IL, USA), which contains 0.01% of a protease and phosphatase inhibitor cocktail (Thermo Scientific). Equal amounts (50 μg) of total proteins were fractionated through SDS-PAGE on a 10% gel and transferred to PVDF membranes (Roche, Basel, Switzerland). The membrane was blocked with 5% milk/Tris-buffered saline plus Tween 20 (TBST) and incubated with primary antibodies against human E2F1 (sc-251), p53 (sc-6243) and PARP-1 (sc-8007) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). HRP goat anti-mouse IgG (Santa Cruz) and HRP goat anti-rabbit IgG (Cell signaling, Danvers, MA, USA) were used for secondary antibody. Immunoreactive bands were visualized using a LAS-3000 Imager (Fujifilm Corporation, Tokyo, Japan). Equal loading was assessed after probing the same membrane with β-actin antibody (Santa Cruz).

7. Detection of $CD133^+$ glioma stem cells

The cells were harvested through enzymatic dissociation and resuspended in PBS supplemented with 2% FBS. To analyze the cell surface markers, 1×10^6 cells were labeled with a allophycocyanin (APC)-conjugated anti-CD133 antibody (clone 293C3, Miltenyi Biotech, Bergisch Gladbach, Germany) for 30 min at 4℃. APC-conjugated mouse IgG2b isotype control antibody was used to exclude nonspecific binding. The cells were washed three times with PBS and subsequently analyzed through FACSVerse flow cytometry (BD Bioscience).

8. Cell migration assay

A scratch wound-healing assay was performed to assess cell migration. The cells were seeded at 5×10^5 in 6-well plates and incubated for 24 h until reaching the appropriate confluence on the day of experiment. An artificial scratch wound was made using a sterile 200-μl pipette tip on the cell monolayer. After the scraping, the floating cells were washed with PBS and fresh medium was added. Differences in the wound width were microscopically visualized at ×40 magnification at three time points (0, 9 and 18 h).

9. Sphere forming assay

After the 3rd mEHT, the single cell suspension was diluted to a

concentration of 2×10^4 cell/ml in serum-free DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 20 ng/ml recombinant epidermal growth factor (EGF) (Invitrogen), 20 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen), and 1X B27 supplement (Invitrogen) and subsequently plated onto ultra-low attachment 24-well plates (Corning). After 5 days of culture, sphere formation was microscopically evaluated using NIS element BR software Version 4.2 to count the number of spheres greater than or equal to 40 μm in diameter, and to measure the size of each sphere.

10. Statistical analysis

Statistical comparisons were conducted using Student's *t*-test with equal variance, Bootstrap *t*-test with 10,000 random repetitions or Poisson Generalized Linear Model. All data are shown as the means ± SEM. Analyses were conducted using R statistical software (v. 3.0.1). *P* values ≤ 0.05 (*) and ≤ 0.01 (**) were considered statistically significant.

III. RESULTS

1. Electro-hyperthermia inhibits proliferation and induces apoptosis in glioma cells

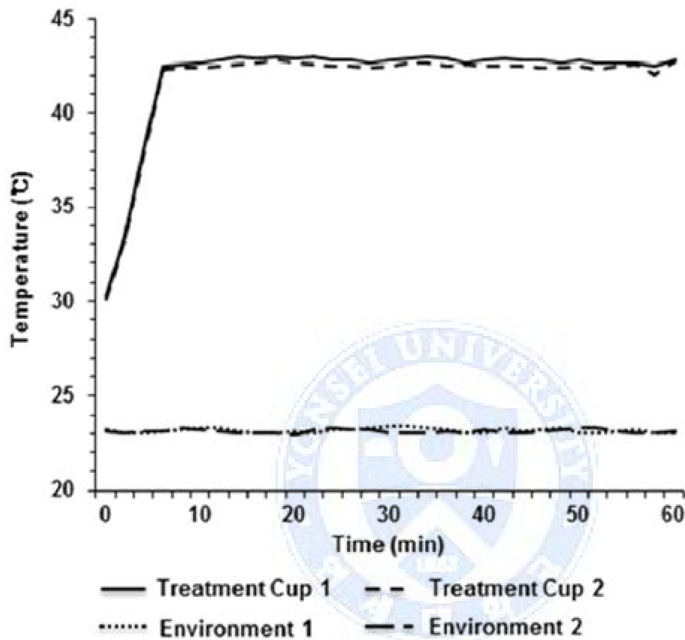

Figure 1. Real-time monitoring of the constant mEHT temperature of 42℃ using sensing probes. Two probes were used to detect the temperature within a Treatment Cup connected to a LAB-EHY 100 device, and the other two probes were placed outside the Treatment Cup to detect the temperature of the surroundings.

As shown in Figure 1, the temperature within a Treatment Cup (a cup containing culture medium and a slide coated with cells) increased to 42℃ within 8 min and remained in a range of 42-43℃ during heating using a LAB-EHY100 device, which controls RF power. To examine the therapeutic efficacy of mEHT, two glioma cell lines, U87-MG and A172, were treated for

1 h with mEHT three times at a 2-day interval, and the cell growth was determined using an MTS assay.

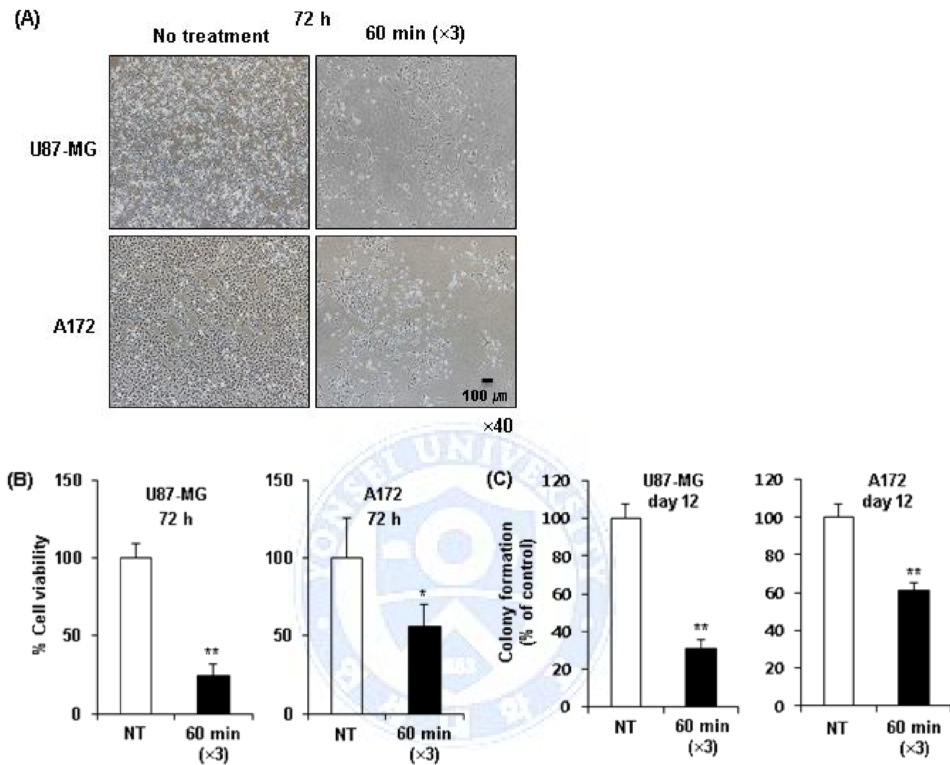

Figure 2. mEHT reduces viability and long-term colony formation in glioma cells. (A) Representative light microscopy images of U87-MG and A172 cells at 72 h after treating three times with mEHT. Scale bar, 100 µm. (B) Measurement of cell viability in U87-MG and A172 cells at 72 h after the third mEHT treatment. The data represent three independent experiments. *NT*, no treatment. **, $P < 0.01$, *, $P < 0.05$ by Bootstrap *t*-test. (C) Observation of long-term colony formation using a clonogenic assay at 12 days after the 3rd mEHT treatment. The data represent three independent experiments. **, $P < 0.01$ using the Poisson Generalized Linear Model.

The mEHT treatments decreased cell proliferation more than 70% and 45% in U87-MG and A172 cells, respectively (Figure 2A-2B). This treatment condition was applied to all subsequent *in vitro* studies. Consistent with the results of the short-term phenotypic assay, mEHT was also effective in inhibiting long-term colony formation (Figure 2C). The magnitudes of the reduction in clonogenicity in response to mETH were similar to the growth inhibition, as determined using the MTS assay (Figure 2B).

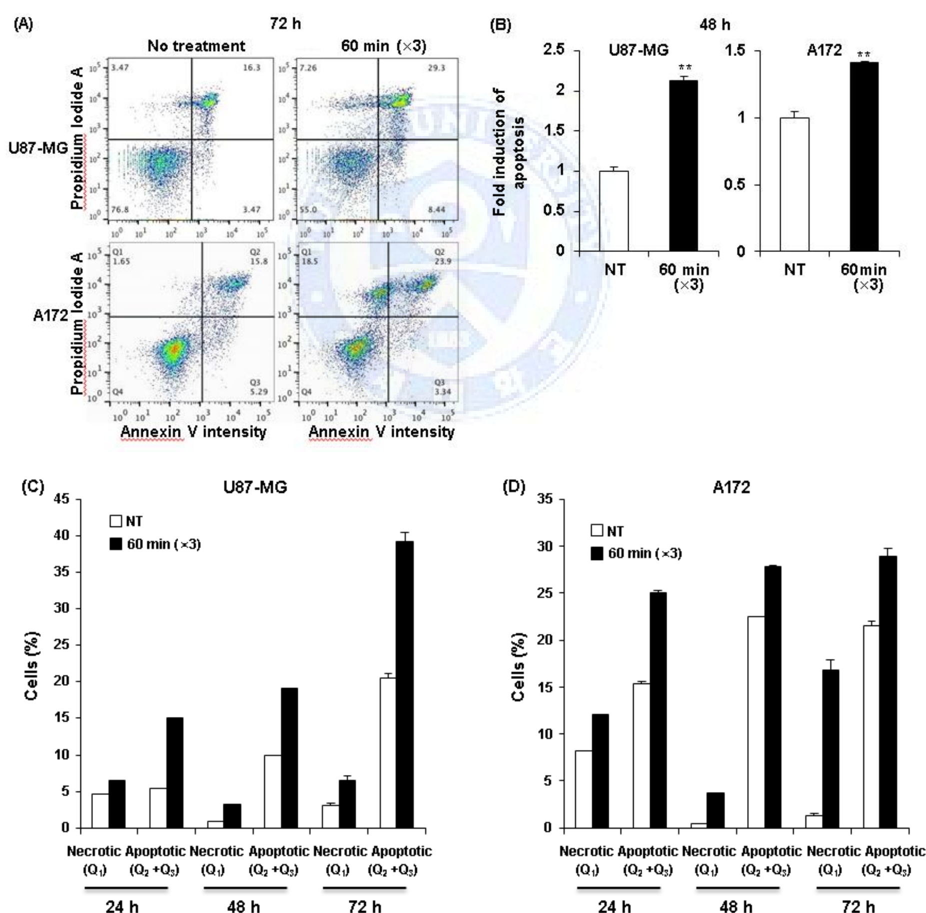

Figure 3. mEHT induces apoptosis in glioma cells.

(A) Detection of the apoptotic cell population through FACS analysis with the intensity of apoptotic marker Annexin V at 72 h after the 3rd mEHT treatment. (B) Detection of apoptosis at 48 h after the 3rd mEHT treatment using the ApoStrand ELISA Apoptosis Detection Kit. The data represent three independent experiments. *NT*, no treatment. **, $P < 0.01$ by Bootstrap *t*-test. (C-D) Detection of the apoptotic cell population through FACS analysis based on the intensity of the apoptotic marker Annexin V in U87-MG (C) and A172 (D) cells at 24, 48 and 72 h after the 3rd mEHT treatment. The data represent duplicate experiments. **, $P < 0.01$ by Student's *t*-test with equal variance.

The growth inhibition of mEHT-treated glioma cells might reflect the strong induction of apoptosis as measured through the detection of the apoptotic marker Annexin V using FACS (Figure 3A). Namely, at 72 h after the 3rd mEHT treatment, the percentage of late-apoptotic (Q2 region) plus early-apoptotic cell populations (Q3) in U87-MG and A172 cells increased 80% and 50%, respectively. To confirm the apoptotic induction through mEHT, we conducted an ELISA assay to detect the denatured single-stranded DNA formed in apoptotic cells but not in the necrotic cells or cells with DNA breaks. The results showed that mEHT significantly increased the apoptotic cell population in U87-MG and A172 cells approximately 2.2- and 1.4-fold, respectively, at 48 h after the 3rd mETH (Fig. 3B). To further observe the onset of apoptosis through mEHT, U87-MG and A172 cells were assayed through FACS analysis at various time points (24, 48 and 72 h) after the 3rd mEHT. As

shown in Figure 3C and 3D, mEHT induced both necrosis and apoptosis at all time points examined in glioma cells. However, this treatment mainly increased the number of apoptotic cells at 24 h, the earliest time point examined. These data suggest that mEHT treatments induce apoptosis in human glioma cells.

2. The molecular mechanisms underlying the growth inhibitory effects of electro-hyperthermia

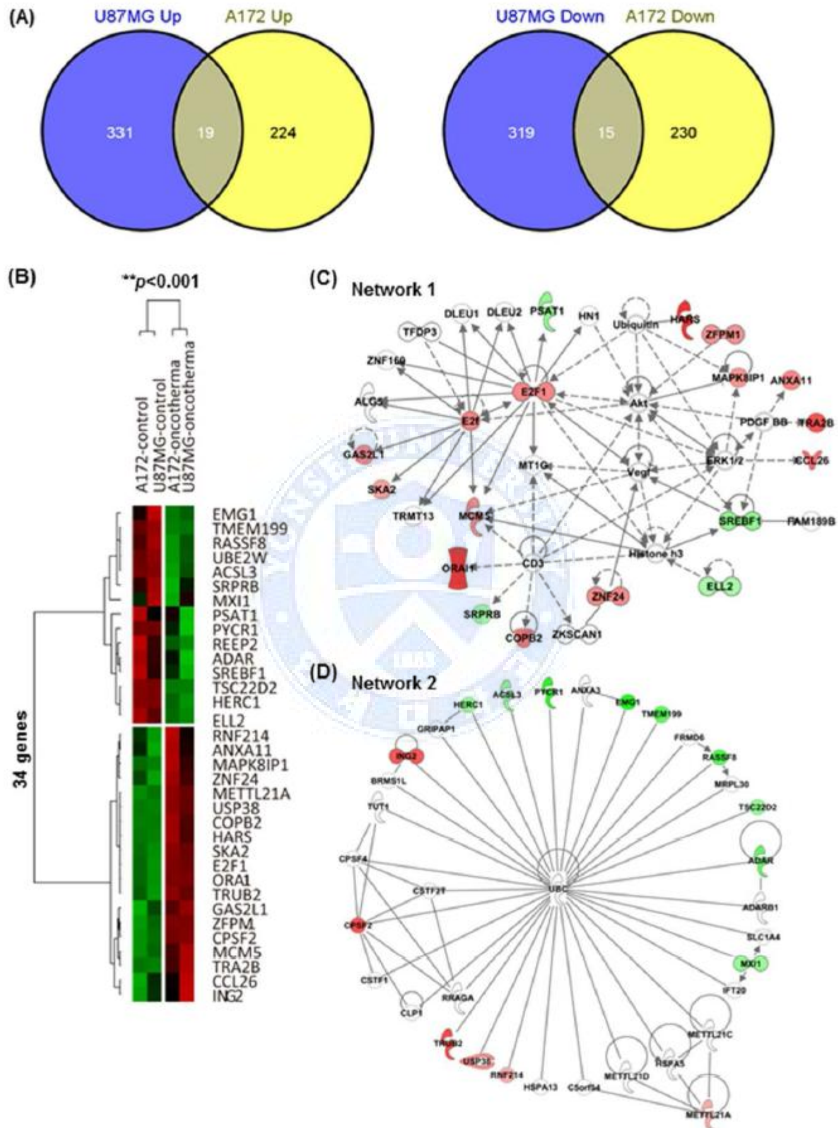

Figure 4. Transcriptomic analysis of gene expression by RNA sequencing following the mEHT treatment. (A) Venn diagrams representing the number of mRNA transcripts up- or down-regulated in U87-MG or A172 cells after mEHT treatment. (B) A heat map of the 34 commonly deregulated genes in U87-MG and A172 cells after normalization to the corresponding sham-treated cells (10,000 repetitions in Bootstrap ANOVA with contrast tests and a threshold cut-off of 2-fold change, **P < 0.001, red (induced) and green (repressed), log2-based scale). (C, D) Two putative top networks with high scores (>19) strongly associated with E2F1 (C) and ubiquitin C (UBC) (D) based on Fisher's exact test (p value < 10-19), indicating a high probability of biological interactions. Up- and down-regulated genes are shown in red and green, respectively. The genes shown in gray are associated with the regulated genes.

To determine the molecular basis of the observed growth inhibition through mEHT, we compared the global gene expression profiles of mEHT-treated U87-MG and A172 cells to those of sham-treated control cells using next-generation RNA sequencing. The paired-end mRNA sequencing analysis revealed that mEHT led to the up- and down-regulation of 684 genes in U87-MG and 488 genes in A172 cells (Figure 4A). Comparing these two gene sets to identify a common anti-cancer mechanism revealed a statistically significant overlap of 34 genes (19 genes up- and 15 genes down-regulated) defined using a Bootstrap *t*-test with 10,000 repetitions and at least a 2-fold change ($P < 0.001$) (Figure 4B), and this effect is considered a common mEHT molecular signature in glioma cells. Ingenuity pathway analysis (IPA)

showed that the 34 mRNA transcripts were functionally enriched in the top two networks shown in Figures 4C and 4D. Consistent with phenotypic changes, mEHT coordinately impacts a restricted number of defined oncogenic pathways acting in concert to decrease cell proliferation and induce apoptosis, providing a rationale for the therapeutic use of mEHT in glioma. Consistently, the expression levels of E2F1 and CPSF2 involved in pro-apoptotic activity and growth suppression were upregulated, whereas two key regulators of cell growth, PSAT1 and ADAR, were repressed. In particular, the transcription factor adenovirus E2 promoter-binding factor (E2F) 1 gene, which is functionally involved in exerting apoptotic progression,[13-15] was upregulated through after mEHT treatment and this process was positioned at a central region of interactive top network 1 (Figure 4C).

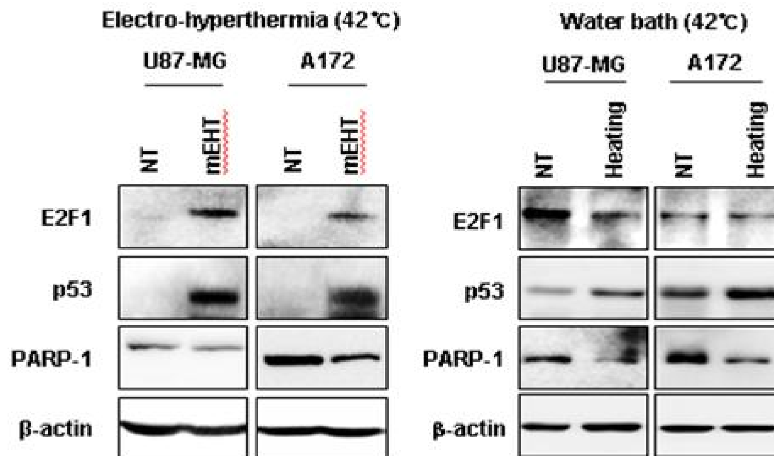

Figure 5. mEHT activates E2F1-mediated apoptotic signaling in glioma cells. Western blot analysis of E2F1, p53 and PARP-1 functionally involved in apoptotic progression. Whole cell lysates were prepared at 72 h after the 3rd mEHT treatment or at 72 h after the 3rd water-bath heating. β-actin was included as a loading control. *NT*, no treatment.

Subsequent Western blot analysis confirmed the increase of E2F1 protein with a concomitant increase of p53 tumor suppressor and decrease of PARP-1, a nuclear enzyme essential for genomic stability and chromatin remodeling in both U87-MG and A172 cell lines (Figure 5). Next, to determine whether E2F1-mediated apoptotic induction was mEHT-specific, we assessed the levels of E2F1, p53 and PARP-1 in glioma cells heated three times for 1 h with a two-day interval in a water bath with 42℃, compared with mEHT-treated cells. As shown in Figure 5 (right panel), while heat stress upregulated p53 and downregulated PARP-1 expression, it did not change the levels of E2F1. This finding identifies E2F1-mediated apoptosis as an essential and common mechanism of growth suppression in mEHT-treated glioma cells.

3. Electro-hyperthermia inhibits glioma growth *in vivo*

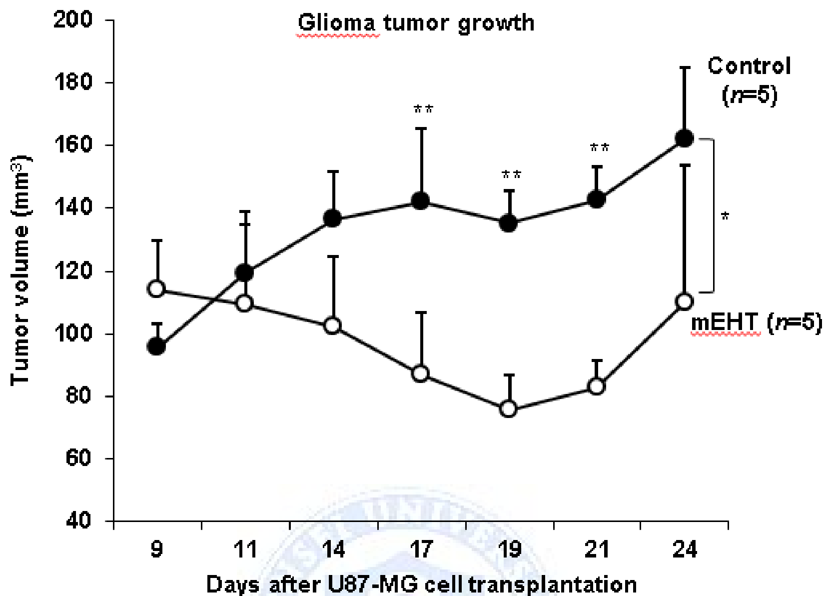

Figure 6. mEHT inhibits tumor growth in mice. Kinetics of tumor growth: mEHT was directly applied to the surface of each tumor after inoculation with 1 × 10^7 U87-MG cells, and the tumor diameters were measured at the indicated days using digital calipers. The results are shown as the mean tumor volume ± s.d. **, $P < 0.01$, *, $P < 0.05$ (n=5 vs. n=5) using Student's t-test with equal variance.

We next investigated whether mEHT could suppress glioma tumorigenicity. To test the therapeutic efficacy of mEHT *in vivo*, we used a U87-MG xenograft in nude mice. All mice received subcutaneous injection of 1×10^7 U87-MG glioma cells in the right flank and on day 9 when tumors had reached an average volume of ~100-120 mm^3, and the mice were randomly assigned to either mEHT treatment or control groups. Electro-hyperthermia

was directly applied 7 times on the surface of each tumor on 9, 11, 13, 15, 17, 19 and 21 days after the glioma cell transplantation, and tumor growth was monitored using a caliper ruler. The results showed that mEHT treatments significantly suppressed the growth of human glioma xenografts (Figure 6) (*$P < 0.05$).

4. Electro-hyperthermia inhibits the maintenance of glioma stem cells

Increasing evidence has indicated that a small fraction of cancer cells are CSCs, responsible for tumor persistence and relapse, metastasis, chemoresistance and radioresistance. As shown in Figure 4B, we observed that mEHT downregulated the adenosine deaminase acting on dsRNA (ADAR, also known as ADAR1) and upregulated the cleavage and polyadenylation specificity factor subunit 2 (CPSF2), both of which are functionally associated with CSC biology. Recent studies have demonstrated that ADAR1 knockdown impaired the *in vivo* self-renewal capacity of chronic myeloid leukemia progenitors,[16] and the loss of CPSF2 expression was associated with an increase in thyroid cancer invasion and the CSC population, particularly upregulating the level of a CSC marker CD133.[17] It has also been reported that glioma CSCs express CD133 and grow in spheroid patterns in special growth medium.[18, 19] Thus, we examined whether mEHT could affect the maintenance of glioma stem cells. To accomplish this, we first measured the

change in the percentage of CSC portion after the 3rd mEHT treatment. Next, 20,000 U87-MG cells treated with mEHT in the same way were plated on a 24-well plate and cultured for 5 days to observe the change in sphere forming capacity by counting the number of spheres greater than or equal to 40 μm in diameter and measuring the size of each sphere.

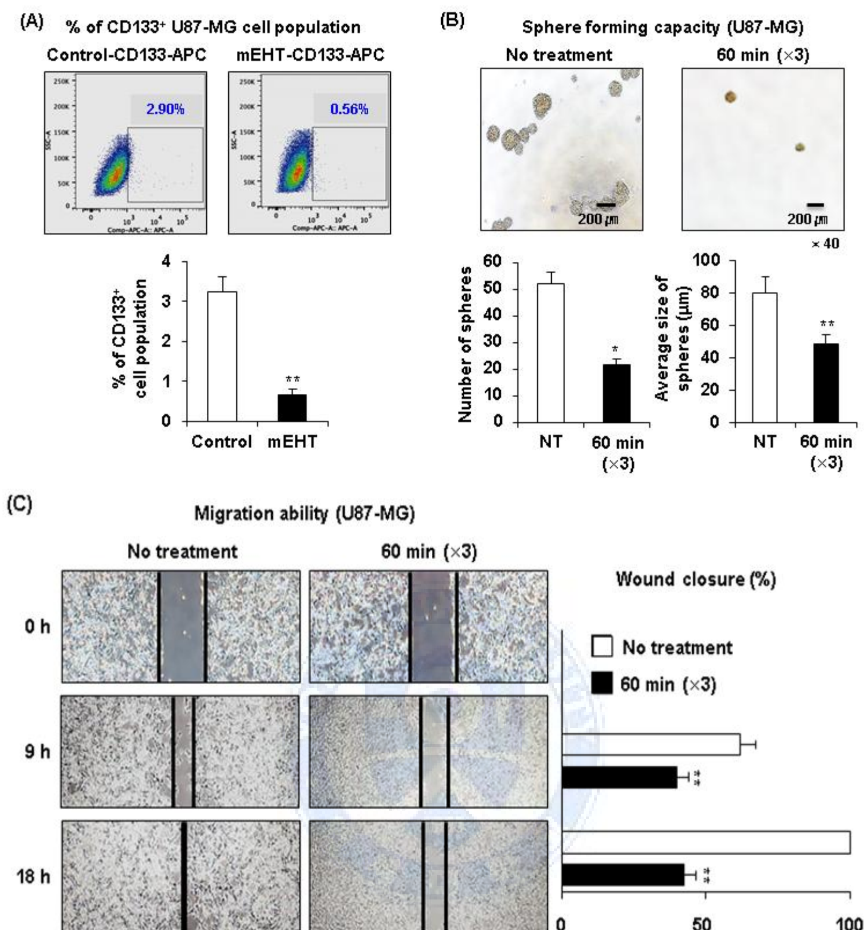

Figure 7. mEHT reduces glioma stemness. (A) Detection of the CD133+ U87-MG cell population through FACS analysis. A total of 1×10^6 cells exposed to the indicated treatments were labeled with APC-conjugated anti-CD133 antibody and subsequently analyzed through flow cytometry. The data represent duplicate experiments. **, $P < 0.01$ by Student's t-test with equal variance. (B) Measurement of the sphere formation. After mEHT or sham treatment, the single cell suspension was diluted to a concentration of 2×10^4 cells/ml and plated onto ultra-low attachment 24-well plates. After 5 days of culture, sphere formation was evaluated using a microscope and NIS element BR software to count the number of spheres greater than or equal to 40 μm in diameter and to measure the size of each sphere. The data represent duplicate experiments. *NT*, no treatment. *, $P < 0.05$ by Student's t-test with equal variance, **, $P < 0.01$ by Bootstrap t-test. (C) Measurement of cancer cell migration ability. Representative images of the migration of sham- or mEHT-treated U87-MG cells in the scratch wound-healing assay. The bar graphs shows the means ± s.d. of gap width of wounds at the indicated time points from three independent assays. **, $P < 0.01$ using the Bootstrap t-test.

As expected, mEHT eliminated the $CD133^+$ U87-MG cell population approximately 80% (Figure 7A) and significantly decreased cancer stemness by reducing both the number (48 spheres formed in no treatment vs. 20 in mEHT) and the average size of spheres (Figure 7B) when compared with control treatments. In addition, the scratch would-healing test showed that mEHT suppressed U87-MG cell migration, indicating the decreased stemness of the cells (Figure 7C). These data indicate that mEHT could prevent glioma recurrence through the elimination of CSCs in the brain tumor microenvironment.

IV. DISCUSSION

mEHT concentrates the modulated radiofrequency current to intercellular electrolytes, generating a temperature gradient between the inside and outside of cells.[5] This gradient destroys the tumor cell membrane, leading to apoptosis or necrosis.[6, 20, 21] Numerous clinical studies have demonstrated that mEHT is effective for controlling advanced primary and metastatic malignancies, such as bone (metastatic), breast cancer and malignant glioma.[10, 22-24] However, the molecular mechanism underlying the induction of cancer cell death through mEHT remains unclear. In the present study, mEHT induced extensive apoptosis in both U87-MG and A172 glioma cell lines (Fig. 3). The phenotypic changes induced through mEHT were associated with the coordinated and common dysre gulation of 34 genes (Fig. 4), as assessed through a global transcriptomic analysis for RNA sequencing, including the upregulation of E2F1, a transcription factor that plays a pivotal role in accelerating apoptotic progression.[13-15] Both the p53 and E2F1 are defective in almost all types of tumors,[25] while U87MG glioma cell line is known to have wild type p53.[26] A previous study showed that overexpression of E2F1 is associated with loss of cell viability and E2F1-mediated glioma cell death is caused by apoptosis.[27] One study prior to that reported the transfer of exogenous wild-type p53 cDNA did not cause apoptosis in cells that express endogenous wild-type p53, which might be from the overexpression of p21, the negative regulator of the cell cycle.[26] Based on them, the upregulation of E2F1 paralleling the increase of p53 tumor suppressor, a

downstream effector of E2F1, and decrease of PARP-1 (Fig. 5A), seems to be providing a mechanism for the mEHT-mediated inhibition of cell cycle progression and induction of apoptosis in glioma cells. This finding is consistent with the recent studies showing that the stabilization of p53 drives apoptosis via the Rb/E2F1 signaling pathway,[15] and the attenuation of PARP-1 increases E2F1 transactivation to inhibit cell cycle progression.[14] The E2F1-mediated apoptosis induced through mEHT might be an essential molecular mechanism explaining the clinical efficacy of mEHT and therefore requires thorough investigation.

Glioblastoma is the most common primary brain tumor, with a high invasive nature. CSCs in glioblastoma are radioresistant and chemoresistant. Thus, targeting the CSCs might be an efficient therapy to prevent tumor recurrence after treatment.[28, 29] A previous study reported that the conventional hyperthermia effectively reduced a portion of breast CSCs in both single treatment and combinatory treatment with metformin.[30] Through whole-genome RNA sequence analysis, we observed in this study that the inhibition of glioma cell growth through mEHT is associated with the downregulation of ADAR1 transcripts, which was functionally validated to increase self-renewal.[16] In addition, the CPSF2 gene, upregulated through mEHT treatment, was correlated with increased CSC invasion when decreased in expression.[17] These molecular clues prompted us to determine whether mEHT could therapeutically eliminate glioma stem cells. The results of various CSC assays revealed that mEHT showed remarkable efficacy in eliminating the $CD133^+$ glioma stem cell population and

decreasing sphere formation and migration. These findings suggest that mEHT could be a novel treatment option for preventing glioma recurrence.

V. CONCLUSION

In conclusion, in the present study using human glioma cell lines, we showed that mEHT induced the inhibition of cell proliferation and clonogenicity, induced apoptosis *in vitro*, and suppressed the growth of a glioma xenograft in nude mice. The anti-tumorigenic effects of mEHT might reflect the upregulation of the E2F1 transcriptional factor. mEHT also eliminated cancer stem cells or stem-cells like glioma cells, indicating that mEHT might be effective to prevent the recurrence and metastasis of tumors.

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ABSTRACT (IN KOREAN)

전기적 온열치료의 신경아교종에 대한 항암효과 기전으로서의 E2F1 매개 세포자멸사

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차 지 혜

목적: Modulated electro-hyperthermia (mEHT)는 고주파를 이용하여 열을 발생시키는 심부 전기적 온열치료의 한 방법으로, 신경아교종을 포함한 여러 종양에서 현저한 치료 효과를 보임이 보고되어 왔다. 본 연구의 목적은 mEHT를 시행 받은 암세포의 표현형 변화와 그 분자기전을 밝히는 데 있다.
재료 및 방법: 신경아교종 세포주 U87-MG와 A172에 mEHT (42℃/60분)를 2일 간격으로 3회 시행하였고 성장 억제 확인을 위해 MTS와 FACS, 현미경적 분석을 시행하였다. mEHT가 일으키는 분자기전 파악을 위해 RNA 서열 결정을 시행하여 mEHT에 의한 유전자 발현 변화를 확인하였다. mEHT의 생체내 작용 확인을 위해 누드생쥐에 U87-MG 세포주의 이종이식실험을 시행하였다.
결과: mEHT는 세포자멸사를 강하게 유도함으로써 신경아교종 세포의 증식을 억제했다. 전사체 분석을 통해 mEHT 시행 후의 증식 억제 효과가, E2F1와 CPSF2의 상향조절과 ADAR과 PSAT1의 하향조절을 비롯한 분자변화에 기인함을 확인하였다. 후속적으로 단백질흡입법을 시행하여 mEHT가 E2F1, p53을 증가시키고 PARP-1을 감소시키는 것을 확인했으며, 이는 세포자멸사를 촉진하는 신호전달체계와의 연관성을 시사하는

결과이다. 또한 누드생쥐 이종이식실험에서도 mEHT는 신경아교종의 증식을 효과적으로 억제하였으며, $CD133^+$ 신경아교종 줄기세포의 비율을 크게 감소시키고 종양세포 이동과 구 형성을 억제하는 작용을 나타냈다.
결론: mEHT는 E2F1이 매개하는 세포자멸사의 촉진을 기전으로 하여 신경아교종 세포의 증식과 세포 이동을 억제하는 것으로 생각되며, 뇌종양의 치료에 효율적으로 적용할 수 있을 것으로 생각된다.

핵심되는 말: 전기적 온열치료, 신경아교종, E2F1, 세포자멸사, 종양줄기세포

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