Evaluation of systemic external microwave hyperthermia for treatment of pleural metastasis in orthotopic lung cancer model

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Abstract. External microwave (EMW) hyperthermia system (2.45 GHz wave frequency) was evaluated by *in vitro* studies and *in vivo* pleural metastasis animal model. Three different non-small-cell lung cancer cells and normal fibroblast cells (control) were treated once a day for 3 days with the prototype EMW system applying mild (39˚C), moderate (43˚C), and severe (47˚C) hyperthermia. On Day-4, tested cells were retrieved and examined by apoptosis assay kit and Western blot analysis. Cancer cells treated with moderate hyperthermia showed significant apoptosis; yet no major damage was observed to normal fibroblast cells. Western blot analysis indicated cleavage on caspase-3, -9 and PARP. Also in the cell cycle analysis, increase of sub G0-G1 population was identified. After optimization of the heating intensity for *in vivo* environment, we created pleural metastatic animal model in 24 immune deficiency mice (male nu/nu mice) to evaluate inhibitory effect of systemic EMW hyperthermia for disseminated tumor growth. Out of 24 mice, 8 received mild and 8 received moderate hyperthermia, and remaining 8 were the no treatment control. Whole chest area of the experimental animals was irradiated 3 times a week for 2 weeks (total of 6 time irradiations). No significant adverse event was observed including abnormal weight loss, skin burn, ulceration, and death. Metastasized tumors around the pleura and chest cavity were 75% reduced in size and weight compared to non-treated control group. Harvested tumors were stained and TUNEL assay demonstrated significant apoptosis in a moderate hyper-thermia group. The EMW hyperthermia system may be possible alternative tool as a systemic hyperthermia therapy in severely advanced lung cancer patients. Further study is necessary to determine device safeness, efficacy, and synergistic effect to other possible combination therapies.

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Introduction

It is generally known that hyperthermia therapies can contribute certain degree of anti-cancer effect. In fact, it was demonstrated that hyperthermia combination therapy could enhance conventional cancer therapies such as chemotherapy by increase of blood perfusion to the tumor, breakage of tumor cell membrane, eventually enhances the drug delivery. Hyperthermia can also affect synergistic impact to radiation therapy by improving radiation sensitivity against cancer cells (1,2).

Electromagnetic wave hyperthermia is one of heating techniques, and various types of magnetic wave-length have been used for internal and external applications such as microwave antenna probes, ultrasound applicator, and radio-frequency electrodes. Even though localization of electromagnetic power should be beneficial for eliminating normal tissue damage surrounding the solid cancer tumor, severely advanced cancer, in particular, disseminated cancer cells in chest cavity and/or abdominal cavity are generally difficult to be treated with local hyperthermia techniques.

Our previous research demonstrated that the external microwave (EMW) hyperthermia induced apoptosis suggesting some efficacies for treating cancer (3). In this study, we further investigated in-depth the systemic EMW hyperthermia in optimization of hearting regimen, in cell cycle and apoptosis pathway analysis, and implications with different temperature levels. Furthermore, orthotopic pleural metastasis lung cancer model was used to mimic the end-stage lung cancer for evaluating efficacy of the EMW irradiation to whole chest/abdominal area.

Materials and methods

Prototype EMW hyperthermia system. A prototype EMW hyperthermia system was constructed by incorporating a 2.45 GHz frequency microwave generator, a 500-watts

power unit, and a controller system. Two of the EMW hyperthermia systems were mounted facing each other into an electromagnetic immune console. Irradiation and interval times were pre-programmed by digital timer. Tested specimens (cultured cells) or animals (mice) were located between two EMW systems (Fig. 1). Temperature inside the cell culture well and animal's core temperature (rectum/esophagus) were monitored by real-time optical thermo sensor (LT-X5 True, IPITEK Corp., Carlsbad, CA, USA). Unlike conventional thermister thermometer, these sensors are absolutely immune against electromagnetic wave such as microwave and allow to measure temperature within ±0.2˚C of accuracy. The sensor probes were connected to personal computer system (IBM ThinkPad T43, Windows XP professional) through the IPITEL 4-channel OEM data acquisition board. Temperature was calibrated based upon the standard temperature probe (HART Scientific, American Fork, UT, USA; Device/1502 serial 8C573, Probe/PRT 5622-05) and recorded every second. Heating procedure was pre-programmed to repeat EMW irradiation 3 times and set intervals in between each heating session for 2 sec. Each of the heating times was changed from 3 up to 9 sec (Fig. 2).

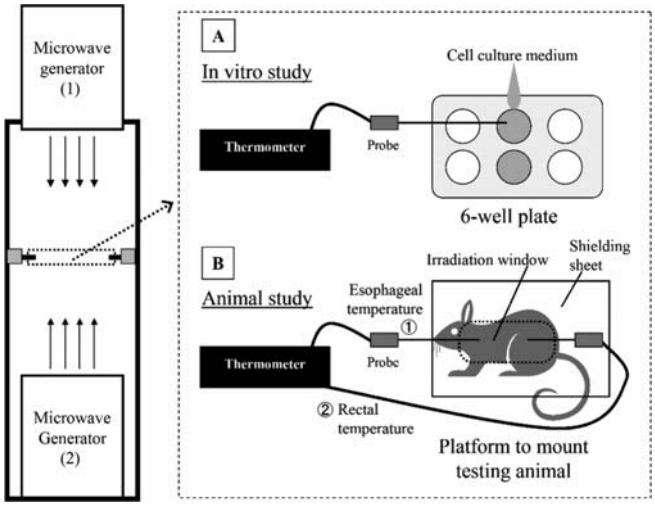

Figure 1. Schematic of external microwave hyperthermia system used for the *in vitro* and *in vivo* studies. (A) Real-time optical fiber temperature probe was placed in the cell culture medium. (B) Real-time optical fiber temperature probes placed in the animal esophagus and rectum.

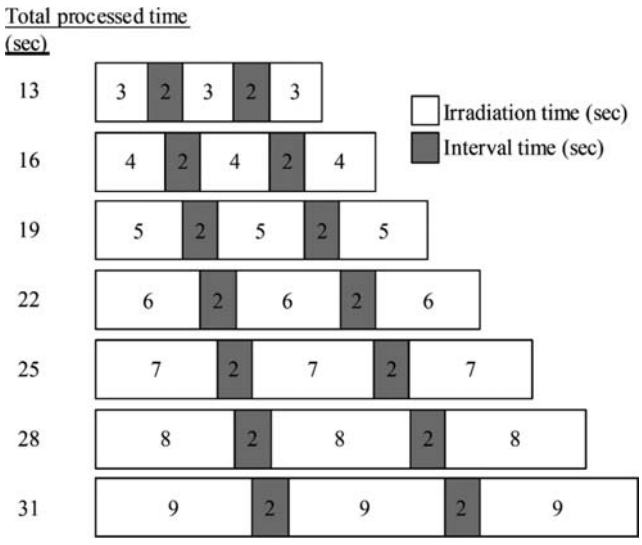

Figure 2. External microwave irradiation procedures in different irradiation regimes. An intermittent irradiation time (white bar) was changed from 3 up to 9 sec, and an interval time (gray bar) was fixed at 2 sec. The left column number represents the total processed time.

Phantom study. To examine dosimetry of the experimental EMW hyperthermia system, physiological phantom model was used. With a standardized technique and materials established by collaborating institution (4), a specific adsorption rate (SAR) was measured. Two testing EMW systems were positioned facing each other to simulate experimental condition inside electromagnetic shielded room, and the physiological phantom was placed in between the testing EMW systems. Immediately after EMW irradiation, front face of irradiated phantom and sagittal cross section were examined by an infrared thermography for converting into the SAR (4).

Cell lines and cell culture. The 3 different human non-small-cell lung cancer (NSCLC) cell lines: H322, H1299 and H460, and normal human lung fibroblast cell line: WI-38, were obtained from American Type Culture Collection (Manassas, VA). The NSCLC cell lines or WI-38 were maintained in RPMI or MEN supplemented with 10% FCS and 5% glutamine.

Cell viability assay. Inhibition of tumor cell growth by treated with the EMW hyperthermia system was analyzed by viable cell counting with a trypan blue exclusion assay. NSCLC H322, H1299, H460 and a normal fibroblast WI-38 cells were seeded in 6-well plates at $2x10^5$ cells/well (Day-0). The following 3 days (Day-1, 2 and 3), the cells were treated with 39˚C (mild hyperthermia), 43˚C (moderate hyperthermia), and 47˚C (severe hyperthermia). On Day-4, the cell viability in each group was determined by cell count under a microscope.

Analysis of apoptosis and sub G0-G1 population. Induction of apoptosis and increase of sub G0-G1 population in tumor cells treated by the EMW hyperthermia were analyzed using an APO-BRDU kit (BD Biosciences PharMingen, San Diego, CA) by flow cytometry (FACS). Briefly, cells were plated in 60 mm dishes at $4x10^5$ cells/dish for 24 h and then irradiated by the mild and moderate hyperthermia for 3 days. After 24 h of the last treatment, cells were harvested and fixed in 1% paraformaldehyde. The BrdUTP-incorporated DNAs were detected using FITC-labeled anti-BrdU antibody and analyzed by FACS. The cells were also stained with propidium iodide/RNase buffer and analyzed by FACS for determination of cell cycle kinetics in the same cell samples.

Western blot analysis. Anti-HSP70, anti-HSP90, Chk2, PTEN, and Bax were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-Phospho-Chk2 (Thr68), anti-AKT,

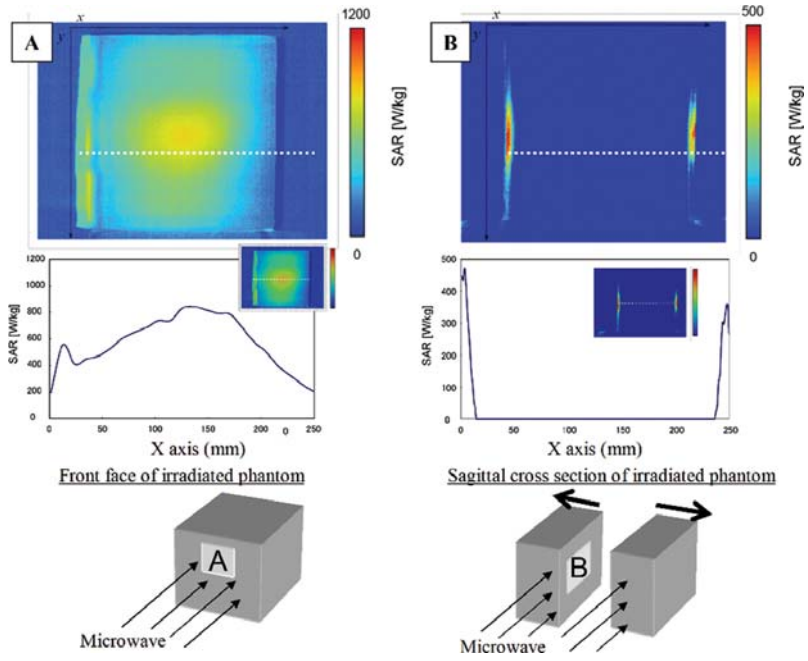

Figure 3. SAR distribution analysis with physiological phantom after external microwave irradiation. (A) Front face of irradiated phantom, (B) sagittal cross section of irradiated phantom. The highest intensity area is presented in red color located in the middle of the irradiation window.

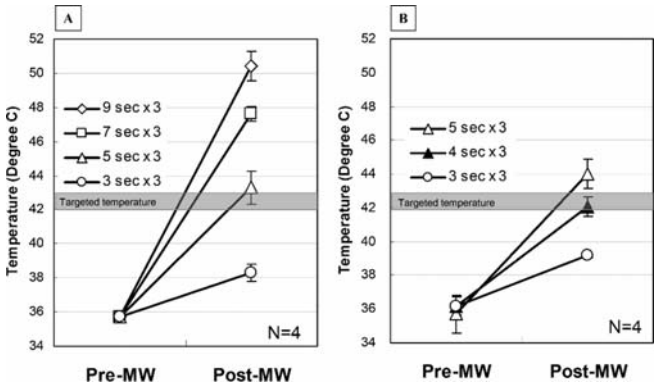

Figure 4. Results of *in vitro* and *in vivo* temperature analysis. (A) *In vitro* analysis with saline solution in a 6-well plate. (B) *In vivo* analysis with BAL/C mice. To protect the experimental animals, severe hyperthermia was not applied. Results were reported as the mean ± SD in 4 tested subjects for each treatment group.

anti-Phospho-AKT (Ser473), anti-Phospho-mTOR (Ser2448), and anti-caspase-3 were from Cell Signaling Technology (Beverly, MA), anti-phospho-histone H2A.X (Ser139) was from Upstate (Lake Placid, NY), anti-Bcl-XL and anti-PARP [Poly (ADP-ribose) Polymerase] were from BD Biosciences PharMingen, anti-BNIP3 and anti-ß-actin were from Sigma (St. Louis, MO). In all Western blots, anti-ß-actin was done as loading control. Cell lysates were prepared form NSCLC H322 cell line, which was irradiated or not with REMW hyperthermia, in lasemini/urea buffer (125 mM Tris pH 6.8, 4% SDS, 10% glycerol, 6 M urea) with proteinase inhibitor (Roche Molecular Biochemicals, Mannheim, Germany). Protein concentrations were assayed using BCA Protein Assay Reagent (Pierce Biotechnology, Rockford, IL). The cell lysates (about 50 μg) were separated by a standard SDS-PAGE and detected by Western blotting.

Animal studies. All animals were maintained and animal experiments were performed according to the provisions of the Animal Welfare Act, PHS Animal Welfare Policy, the principals of the NIH Guide for the Care and Use of Laboratory Animal and institutional guidelines established by the Animal Core Facility of Baylor College of Medicine. The animal protocol, which includes animal housing, experimental procedure, analgesia management, euthanasia, post-experiment autopsy, and other necessary humane care, was approved by Baylor College of Medicine, Institutional Animal Care and Use Committee. A human NSCLC H322 orthotopic lung cancer mouse model was used to evaluate the therapeutic efficacy on tumor growth by the systemic EMW hyperthermia irradiation. The animals used in this study were male nu/nu mice (4-6 weeks of age) that were purchased from Charles River Laboratories (Wilmington, MA). To establish orthotopic pleural tumors, mice were inoculated with $2x10^6$ H322 cells in 100 μl PBS by intra-thoracic injection with a 27-gauge needle described previously (5). Those mice were randomly divided into 3 groups (8 mice/group); Control, mild hyperthermia, and moderate hyperthermia group. Orthotopic tumors are usually established on the lung or on the inner thoracic membrane 10-14 days after tumor inoculation. Animals were systemically treated either by mild or moderate EMW hyperthermia on Days-12, 13, 14, 19, 20, and Day-21 after tumor inoculation. Seven days after the last treatment, all mice were sacrificed humanely and the total number and total weight of pleural tumors of each mouse were examined. Furthermore, to determine induction of apoptosis, the pleural tumors (larger than 5 mm) from each group were randomly harvested and freshly frozen. Induction of apoptosis was

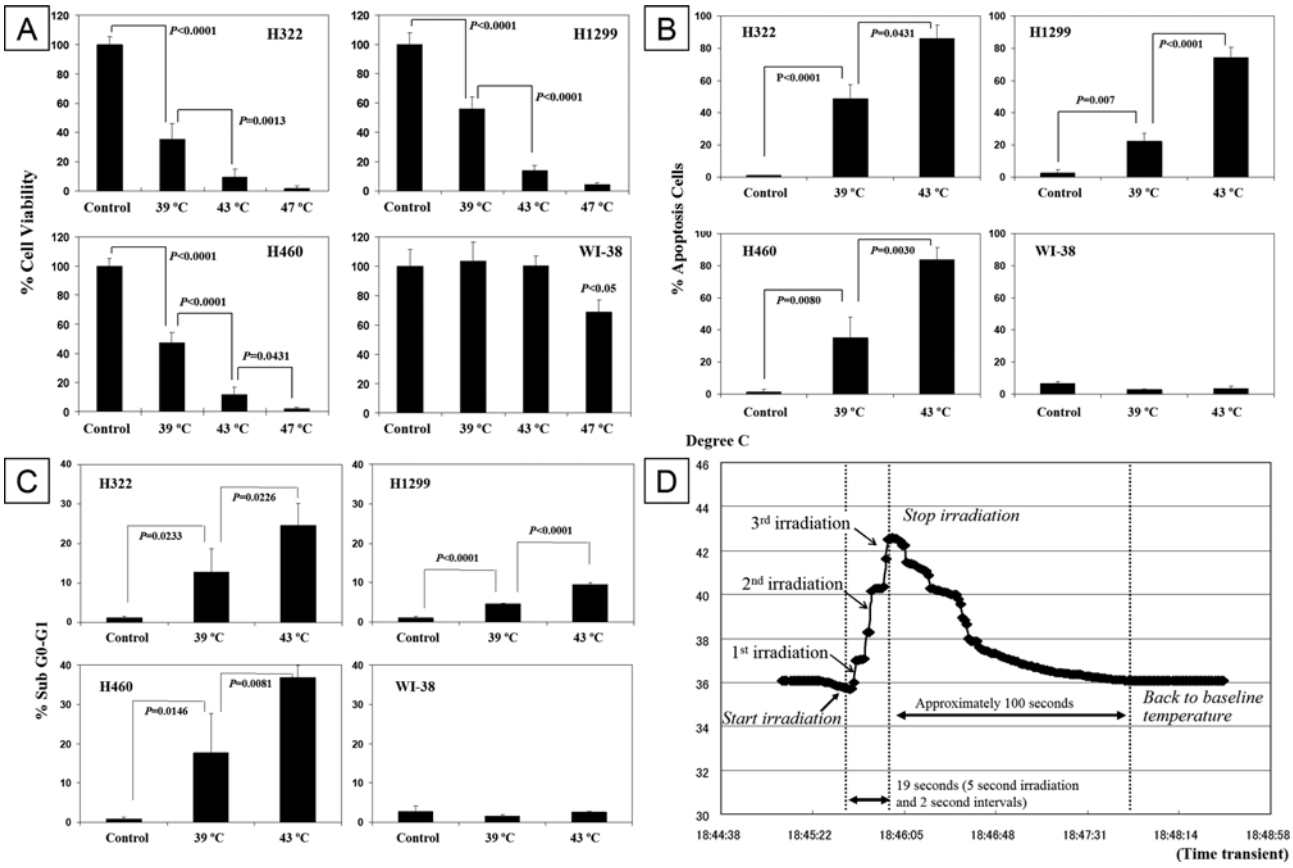

Figure 5. NSCLC H322 cell lines were treated with the EMW hyperthermia system at 39, 43, and 47˚C (only cell viability study) and compared with control group (37˚C). The percent cell viability in each treatment group was determine by viable cell count and calculated relative to those untreated cells as 100% (A). (B and C) Induction of apoptosis and increase of sub G0-G1 population were analyzed using an APO-BRDU kit by FACS. Black solid bars and error bars indicate mean and SD value, respectively, in 3 independent subjects for each treatment group. The significance of differences between treatment groups was analyzed by the ANOVA and Fisher's test.

analyzed using *In Situ* Cell Death Detection kit, using TUNEL staining with FITC-labeled dUTP (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instruction. The fluorescence images were examined under a fluorescence microscope equipped with a digital camera and imaging analysis software.

BAL/C mice (4-6 week of age) were purchased from Charles River Laboratories (Wilmington, MA) and used only for *in vivo* temperature analysis prior to *in vivo* studies with orthotopic pleural metastasized nu/nu mice.

Statistics. All the experiments were repeated at least 2 times with duplicates or triplicates of samples. ANOVA and Fisher's test was used to compare the values of the test and control samples, and $P<0.05$ was considered statically significant. StatView 5.0 software (Abacus Concepts, Inc., Berkeley, CA) was used for all of the statistical analyses.

Results

Dosimetry of the experimental EMW hyperthermia system. Fig. 3 shows a typical SAR distribution of the EMW hyperthermia irradiation tested by a physiological phantom model. Highest intensity was in the center of irradiation window, and the irradiated microwave beam was adsorbed and attenuated within 1.5-2.0 cm depth of the phantom. Based upon this result, the highest EMW intensity area was positioned to the testing subject prior to *in vitro* and *in vivo* experiments.

In vitro and in vivo temperature analysis. *In vitro* study indicated that 5-sec irradiations (repeated 3 times) and 2-sec intervals (total of 19 sec processed time) was the optimal testing condition for adjusting temperature of cell culture medium into the targeted temperature window between 42 and 43˚C (Fig. 4A). *In vivo* study demonstrated that relatively short irradiation time, 4-sec irradiations (repeated 3 times) and 2-sec intervals (total of 16 sec processed time) was the optimal condition for testing animals to achieve moderate hyperthermia (Fig. 4B).

Inhibition of tumor cell growth and induction of apoptosis by the EMW hyperthermia. To test inhibitory effect of tumor cell growth with the EMW hyperthermia treatment, we evaluated the cell viability at various temperatures by the EMW hyperthermia machine in 3 different NSCLC cell lines and 1 normal lung fibroblast cell line (Fig. 5A). For cell viability analysis of 3 NSCLC cell lines (H322, H1299 and H460), mean value of percent cell viability based upon the

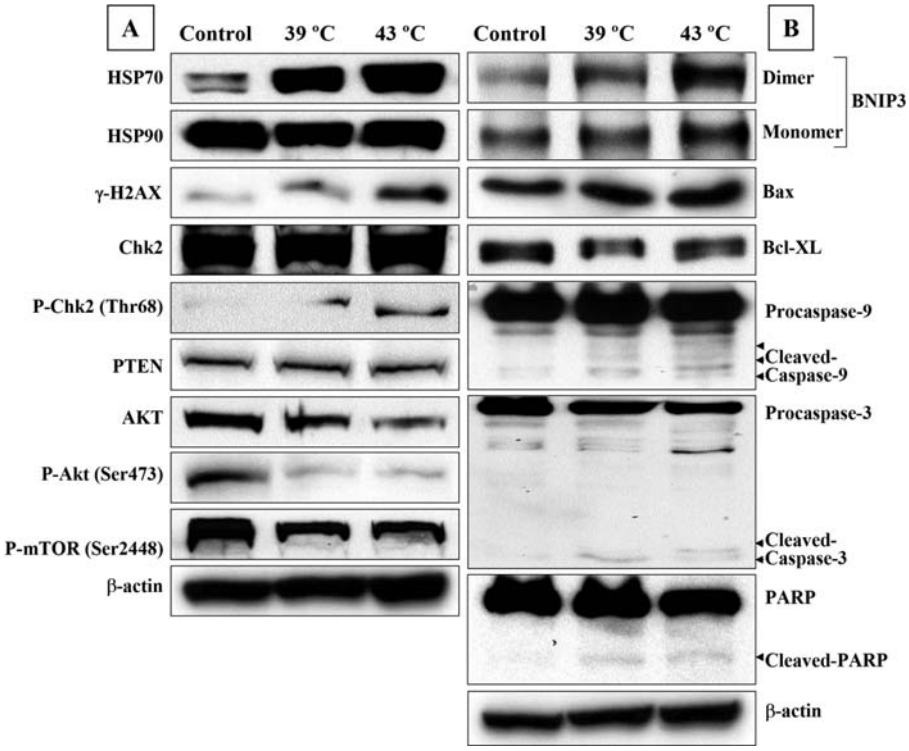

Figure 6. NSCLC H322 cells were irradiated with the EMW hyperthermia system at 39, and 43˚C for 3 days, and untreated cells were used as negative controls. After 24 h of the last irradiation, effects of the EMW hyperthermia on heart shock proteins (HSP70, 90), DNA damage pathway (γ-H2AX, Chk2, and Phospho-Chk2), AKT-mTOR pathway (PTEN, AKT, Phospho-AKT, and Phospho-mTOR) (A), and Bcl-2 family (BNIP3, Bax, and Bcl-XL) (B) were analyzed by Western blotting. ß-actin was used as a loading control. The activation of caspase-9, -3 and PARP was detected by the cleaved fragment bands form the pro-proteins (B).

control (100%) was 35.5% (39˚C), 9.6% (43˚C), and 1.9% (47˚C) in H322, 55.9% (39˚C), 13.8% (43˚C), and 4.2% (47˚C) in H1299, 47.3% (39˚C), 11.9% (43˚C), and 2.1% (47˚C) in H460 ($P<0.0001$). On the other hand, EMW hyperthermia did not affect cell viability of normal lung fibroblast cells, WI-38 with mild or moderate hyperthermia. Only overheated severe hyperthermia group (47˚C) indicated 68.6% cell viability in WI-38. To further assess induction of apoptosis in only tumor cells by the EMW hyperthermia, we examined %TUNEL positive cells and %sub G0-G1 phase cells by FACS analysis using an APO-BRDU kit (Fig. 5B and C). The induction of apoptosis and the accumulation of sub G0-G1 were dramatically increased in temperature dependent manner in H322, H1299, and H460 treated with EMW hyperthermia, respectively, compared to untreated those cells ($P<0.01$). On the other hand, apoptosis and sub G0-G1 phase were not induced in normal lung fibroblast cells, WI-38 after the EMW irradiation. These results suggested that the EMW hyperthermia therapy enhanced the inhibition of tumor growth associated with cell apoptosis remarkably induced, in particular, by moderated hyperthermia. Mean value of percent apoptosis treated by the EMW moderate hyperthermia was 86.0%, 74.2%, 83.9%, and 3.7% in H322, H1299, H460, and WI-38, respectively. Those results were NSCLC cell-specific, yet did not showed cytotoxicity for WI-38 (normal cells) temperature below 43˚C. Fig. 5D shows a typical throughout transit of the subject temperature from the baseline temperature, peak temperature, and returning to the baseline value. Since the EMW was repeatedly irradiated 3 times in a short period of time, the temperature increased into the targeted therapeutic range simultaneously after irradiation. Then it gradually decreased into the baseline value approximately in 100 sec (usually less than 2 min). With this stepwise heating and interval regimen, the temperature was accurately controlled throughout the entire *in vitro* experiments.

EMW hyperthermia induces apoptosis via activation of DNA damage pathway and inhibition of AKT-mTOR pathway. As shown in Fig. 6, we examined the influence on heat shock proteins such as HSP70 or 90, in H322 cells treated with EMW hyperthermia, which increased the expression of HSP70 but did not show significant influence to HSP90. It has been reported that expression of HSP70 in response to hyperthermia induces antitumor immunity (6), and HSP90 has been specifically involved in the maintenance of the correct conformation of several intracellular proteins and much of them are kinases involved in the control of cell proliferation and survival such as Raf-a and AKT (7). Double-stranded DNA breaks (DSBs) induced by γ-irradiation or various chemotherapeutic agents activates the DNA damage pathway through phosphorylation of several molecules, including Histone H2AX, Chk2, and Chk1 (8), and such checkpoint mechanisms allow the cell time to repair the DNA damage before cell cycle progression is resumed, or, if the damage is too extensive, they trigger apoptosis or cellular senescence (9). AKT-mTOR pathway, which is activated in many types

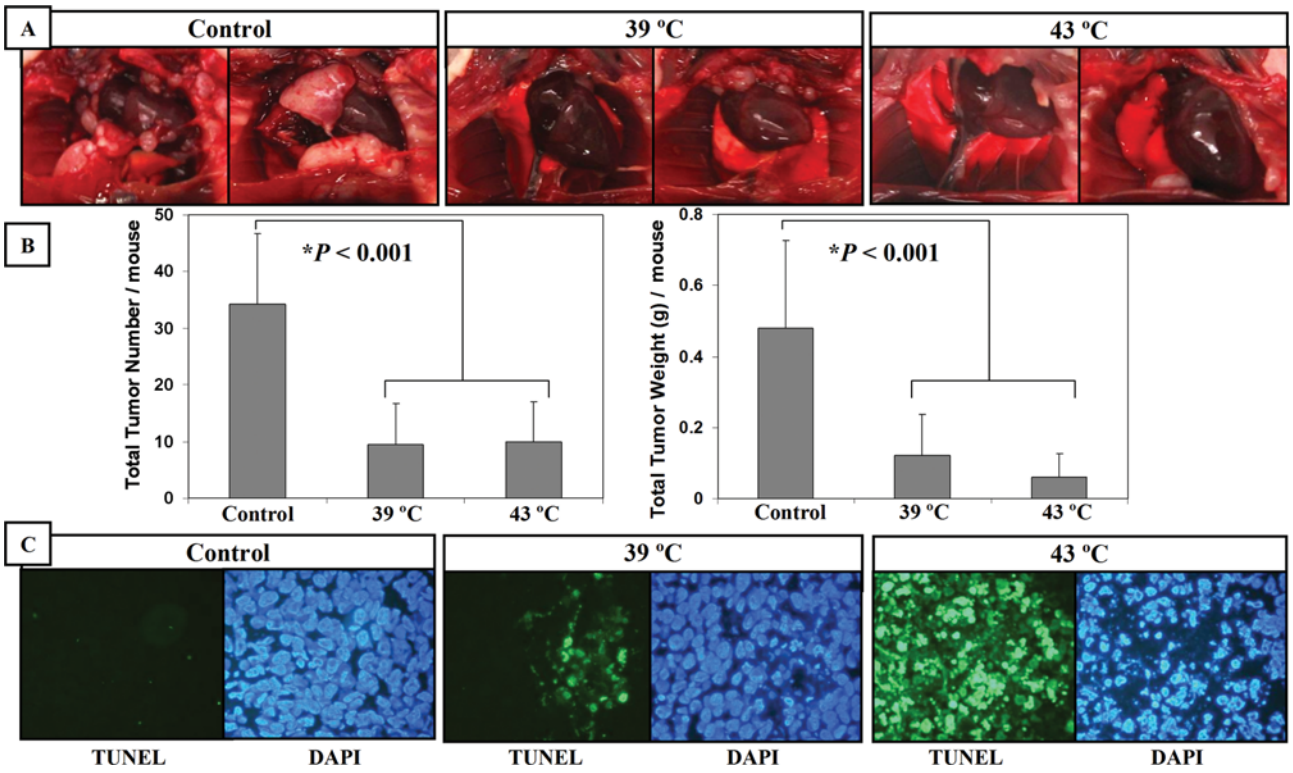

Figure 7. Effect of the EMW hyperthermia cancer therapy in human H322 orthotopic lung cancer mouse model. Experimental animals were treated with mild (39˚C) and moderate (43˚C). Treatment groups, irradiated doses, and schedules were described in detail in Materials and methods section. (A) Autopsy photographs, (B) total number and total tumor weight of resected tumors inside the chest cavity, (C). Apoptosis in frozen tumor tissue sections was detected using an In Situ Cell Death Detection kit by TUNEL staining (TUNEL, bright green) and examined under a fluorescence microscope equipped with a digital camera (Nikon Corp, Tokyo, Japan), and a nuclear staining DAPI (blue) in the same tissue sections were also shown. Magnifications, x400.

of cancer (10,11), is important in apoptosis, since phosphorylation of AKT inactivates the proapoptotic factors (12,13) and activates the mTOR which phosphorylates the downstream molecules leading to apoptosis (14). It was also recently reported that DSBs are involved in heat-induced cell killing for foci formation of γ-H2AX (phosphorylation of histone H2AX at serine 139) (15), and microwave electromagnetic field at 1.95 MHz inhibits the expression of AKT (16). γ-H2AX and phospho-Chk2 increased and total AKT, phospho-AKT, and phospho-mTOR decreased in cells irradiated with the EMW hyperthermia in temperature dependent manner compared to control cells, on the other hand, total Chk2 and PTEN is at the same level in cells of all treatments, suggesting that the EMW hyperthermia remarkably activated the DNA damage checkpoint pathway and inactivated the AKT-mTOR pathway. Next, we examined whether the EMW hyperthermia treatment influences the Bcl-2 family of proteins by Western blotting. This treatment increased the expression of the dimer, but not the monomer form of BNIP3, whereas control group did not show any influence on either form of BNIP3. Most of the Bcl-2 family proteins such as BNIP3 work with dimer formation (17). Moreover, apoptotic or antiapoptotic Bcl-2 family member Bax or Bcl-XL increased or decreased in temperature-dependent manner. Increased activation of caspase-3 and protease PARP was clearly detected in H322 cells treated with the EMW hyperthermia, compared to non-treated control group. These results suggest that the strong induction of apoptosis in cells treated with the EMW hyperthermia are mediated both by the activation of caspase cascade and apoptotic regulation of Bcl-2 family.

Efficacy study of the EMW hyperthermia in a human H322 lung cancer mouse model of pleural tumors. Based on these *in vitro* studies, we investigated the efficacy of EMW hyperthermia therapy by suppressing tumor growth in a human NSCLC H322 utilizing orthotopic lung cancer mouse model (Fig. 7). To establish orthotopic pleural tumors, mice were inoculated with H322 cells by previously established intrathoracic injection procedures (5,18). The mice were randomly divided into 3 groups (8 mice per group) as follows: Control group (no treatment), mild hyperthermia group, and moderate hyperthermia group. The animals were treated six times in total with EMW over 2 weeks after tumor inoculation. On day 28 after tumor inoculation, we sacrificed all mice and observed the thoracic cavity. As shown in Fig. 7A, gross examination of the autopsy showed extensive tumor growth inside the thoracic cavity in control mice. On the other hand, in mild and moderate hyperthermia group, tumor growth was significantly suppressed and only few small tumors existed in the thoracic cavity. For further in-depth quantitative analysis, all of the tumors developed inside the chest cavity were resected from each mouse and the total tumor number/mouse was counted, and the total tumor weight/mouse was

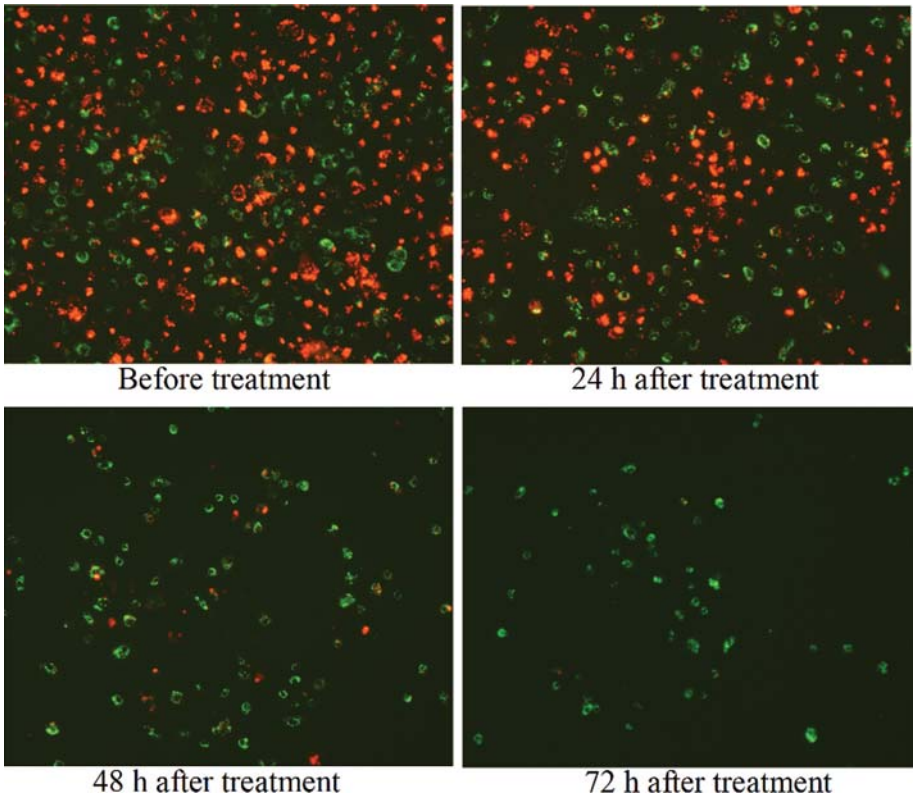

Figure 8. Normal HBE cells and H322 cells were equally mixed. After mixed cells were seeded into a 6-well plate, testing well plate was treated by the same moderate hyperthermia procedure applied for *in vitro* cell viability study. Qtracker® Cell Labeling kit was used for this cell specificity study. Even though equal number of cells were seeded, H322 NSCLC cells gradually decrease (red) after 24, 48 and 72 h. In contrast, normal HBE cells (green) remained at baseline or showed minimal decrease.

measured. As shown in Fig. 7B, the total number and weight of pleural tumors remarkably decreased in the mouse group irradiated by mild or moderate hyperthermia, compared to control mice ($p<0.001$). However, differences of tumor suppression between these two groups were not significant. Furthermore, we analyzed the induction of apoptosis by In Situ Cell Death Detection kit, in pleural tumor cells of each group (Fig. 7C). Induction of apoptosis in the tumors, as indicated by the bright green fluorescein TUNEL staining, was detected in animals irradiated with EMW hyperthermia, but not in control animals. A much higher degree of apoptosis induction was registered in tumors irradiated at by moderate hyperthermia, as indicated by an extended and intensified TUNEL staining.

Discussion

As well as the results of *in vitro* studies, *in vivo* studies showed significant inhibitory effect for disseminated tumor growth inside the chest cavity by systemic EMW irradiation. Only if the lung cancer is treated with appropriate temperature range (moderated hyperthermia), the EMW may be theoretically effective for inducing NSCLC cell-specific apoptosis as proven by our major apoptosis pathway analysis. However, since it is difficult to measure the temperature at the multiple tumor spot disseminated in the chest cavity, we referenced the core temperature as a heating parameter. Both of the rectal and esophageal temperatures were measured only for initial few cases. It revealed that esophageal temperature showed approximately 1˚C higher than rectal temperature. Since rectal temperature can be more safely and quickly measured rather than esophageal temperature, we routinely monitored and recoded the rectal temperature for estimating esophageal temperature. This is one of major issues on this study and technology limitation. It is practically difficult to measure metastasized tiny tumor temperature, but the core temperature measurement may be reasonable parameter for applying equal microwave intensity to the whole chest cavity.

Conventional microwave with 2.45 GHz wave frequency has been widely used for food heating and/or other industrial applications. As shown on the phantom SRA analysis conducted, majority of the EMW beam was attenuated within few centimeters and seems very little heating effect remained around the deep location with the current short pulse heating procedures. Whole chest area of the small animals such as mice that was used for this series of studies can be easily heated within 20 to 30 sec. However, a large animal involves much thicker subcutaneous muscle and fat tissues, larger volume of the organs compared to small animals (i.e. mice); hence it is difficult for the irradiated microwave beam to reach the targeted cancer tumors, which were generally developed at deep and multiple anatomical locations. Further investigation using different wave-length and heating regimen will be needed prior to large animal experiments.

We also investigated another moderate hyperthermia reproduced by normal hot bath by simulating same targeted

temperature (43˚C) and total requiring time. However, despite temperature and transit time parameters maintained the same, cell viability was not affected compared to control group, and no apoptosis phenomenon was seen (data not shown). This supplemental experiment suggested that the degree of temperature is essential parameter but, most importantly, electromagnetic wave has a key role in inducing apoptosis and other anti-cancer effects. Furthermore, to evaluate the device efficacy, other cancer cell lines (breast cancer and gastric cancer) need to be tested. Mechanism of the cancer cell specificity for the EMW is still unclear. According to our preliminary *in vitro* study, we measured nitric oxide (NO) by using precision NO measuring system (InterMedical Corp., Tokyo, Japan) with NO meter (NO-501), a counter electrode (NOR-20) and a working electrode (NOE-47), before and after the EMW irradiation in W-38 cells and H322 NSCLC cells (19). In contrast to WI-38, which showed no sign of NO generation from the cell culture wells after the EMW irradiation, simultaneous surge of NO was detected in H322 wells immediately after the EMW irradiation (data not shown). This result certainly does not provide sufficient scientific evidence to reinforce the cell specificity mechanism, but may give some clues suggesting implication with microwave hyperthermia, cell-based NO induction, and cancer cell apoptosis (20-22). As shown on Fig. 5A-C, no negative effect was observed for WI-38 normal cells with moderate hyperthermia (43˚C) utilizing the EMW. On the other hand, NSCLC cells (H322, H1299, H460) showed cytocidal effect in cell viability, apoptotic effect, and cell cycle alteration. To visualize this cell specificity phenomenon, we stained normal cell in green and stained H322 cancer cells in red (Qtracker® Cell Labeling kit, Molecular Probes, Inc., Eugene, OR, USA), then mixed them together and applied the moderate EMW hyperthermia (Fig. 8). This visualization study indicated that, even in *in vitro* environment, which does not involve micro-vascular circulation, the EMW irradiation only affect cancer cells, but has no or minimal effect on normal cells. These *in vivo* results suggest that the EMW hyperthermia may be a potential therapeutic option for treating disseminated lung cancer in future.

Acknowledgements

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