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Monitoring of Heat Shock Response and Phenotypic Changes in Hepatocellular Carcinoma After Heat Treatment

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Abstract. *Background/Aim: Local recurrence of hepatocellular carcinoma (HCC) after thermal coagulation therapy may be associated with an aggressive phenotypic change. This study focused on the thermal effects on HCC cells and evaluated the heat shock response and phenotypic changes after heat treatment. Materials and Methods: HepG2 and HuH7 cells were used. After heat treatment at 37-50˚C for 5-30 min, we assessed their survival rate, induction of heat shock protein (HSP)70 promoter, proliferation rate, induction of the epithelial–mesenchymal transition (EMT) and cancer stem cell (CSC)-related markers. Results: Induction of HSP70 promoter per surviving cell was maximized after 10 min of heat treatment at 48˚C. Induction of EMT and CSC-related markers was also observed. Conclusion: Sub-lethal heat treatment causes large heat shock response to surviving HCC cells and induce EMT-like and CSC-like phenotypic changes that might contribute to increased aggressiveness.*

Hepatocellular carcinoma (HCC) is the most common primary liver tumor and a leading cause of cancer-related death worldwide (1). Surgical resection is the standard treatment modality for HCC, but its use is usually limited because the majority of patients have associated severe liver dysfunction due to underlying chronic inflammation and cirrhosis. As the most widely used non-surgical treatment approach for HCC, radiofrequency ablation (RFA) has numerous advantages, including its therapeutic effect, repeatability, and safety (2). RFA has been established as an alternative treatment for elderly patients with HCC because of its excellent antitumor effect and its advantage of being less invasive, has lower perioperative risks and fewer deteriorative effects on liver function than surgical resection (3). However, local recurrence and tumor seeding after RFA remain major problems. The possible mechanisms of HCC recurrence after RFA are direct seeding, transvessel, transportal and incomplete ablation, and explosion (4). It has been reported that 4.5% of patients show rapid intrahepatic spread of HCC after RFA (5). Tajima *et al.* reported that epithelial–mesenchymal transition (EMT) and dedifferentiation was observed in resected specimens of locally recurrent HCC after RFA (6, 7). It is suspected that the thermal effect of incomplete RFA may increase the malignant potential of residual tumor cells. In this way, RFA has the significant drawback of limited ablative margins, which is associated with high risk of marginal recurrence (8).

EMT results in epithelial cells becoming spindle shaped, with loss of cellular polarity similar to mesenchymal cells. These phenotypic changes closely correlate with increased cellular motility, invasion and therapeutic resistance (9). The up-regulation of heat shock protein (HSP)70 is relatively common in human tumors, and is often associated with an enhanced resistance to chemotherapy, heat stress and a poor patient prognosis (10). HSP70 may play an important role in the EMT phenotype because it represents an indicator of malignant potential and could discriminate the malignant degree of HCC (11). The EMT has also been reported to endow cells with stem cell-like properties (12). Cancer stem cells (CSC) are specific undifferentiated tumor-initiating cells, have the ability to self-renew, propagate and differentiate leading to cancer growth and progression (13). Likewise, CSCs display aggressive characteristics including increased invasion, metastatic ability and resistance to therapy and predict poor patient prognosis. A number of functional stemness markers in HCC have been identified and characterized such as CD44, CD133 and CK19 (14, 15).

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The present study analyzed the heat shock response of HCC by evaluating induction of the HSP70 promoter under various conditions of heat treatment and investigated the relationship between heat treatment and phenotypic changes of residual HCC cells.

Materials and Methods

Cell culture. Highly differentiated human HCC cell lines, HepG2 and HuH7, were provided by the Department of Human Pathology, Kanazawa University. All cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Nichirei Biosciences, Tokyo, Japan), 100 U/ml penicillin, and 100 μg/ml streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) in a humidified atmosphere of 5% CO_2 at 37˚C. The medium was changed 2-3 times/week.

Heat treatment. Heat treatment was simulated *in vitro* according to a previously reported procedure (8). Briefly, an adherent monolayer of HepG2 and HuH7 cells were grown to 70% confluence, trypsinized, washed in DMEM, collected in 1.5-ml microtubes in 1 ml of medium (5×10^5 cells), and immediately exposed to heat shock using a dry bath incubator (Programmable Cool-Hotter; MAJOR SCIENCE, Saratoga, CA, USA) at the target temperature (37˚C, 44˚C, 46˚C, 48˚C, and 50˚C) for each exposure time. Then, cells were seeded into 60 mm collagen coated dishes in 3 ml of DMEM with 10% FBS and maintained at 37˚C. DMEM was exchanged twice a day to remove debris and dead cells.

Trypan blue exclusion test. After heat treatment, cells were plated in dishes in medium supplemented with 10% FBS for 24 h. Cells were harvested by trypsinization. Then cells were stained with a 0.4% trypan blue solution and counted on an automated cell counter (EVE-MC; NanoEnTek, Seoul, Republic of Korea).

Transfection and luciferase reporter assays. To monitor the heat shock response of HCC cell lines after heat treatment at various temperatures for each time duration, we used the reporter plasmid pDrive5Lusia-hHSP70 (Invivogen, San Diego, CA, USA), which is composed of heat induced human HSP70 promoter and a coelenterazine-utilizing secreted luciferase reporter gene. HepG2 and HuH7 cells were transfected with the reporter plasmid pDrive5Lusia-hHSP70 using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's instructions. Transfected cells were generated from these initial transient transfections by selecting the cultures under 50 μg/ml Zeocin (Invivogen, San Diego, CA, USA). HepG2-L (HepG2-pDrive5Lusia-hHSP70) and HuH7-L (HuH7-pDrive5Lusia-hHSP70) were collected in 1.5 ml microtubes in 1 ml of medium (1,000 cells) and exposed to heat treatment as described above. Then, cells were seeded into 24-well plates and maintained at 37˚C for 8 h. A total of 20 μl of medium sample was mixed with QUANTI-Luc luminescence assay reagent (Invivogen) in a white 96-well plate. Then the concentration of secreted Lucia luciferase was immediately measured using a luminometer (TriStar LB941; Berthold Technologies, Bad Wildbad, Germany).

Cell proliferation assay. Cell proliferation was measured by the 3-(4, 5-dimethylthiazol2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were seeded in 96-well plates with 5×10^3 cells/well and cultured for the indicated times. At the end of experiment, MTT solution (final concentration 0.5 mg/ml) was added to each well and incubated for 4 h. Then, 150 μl DMSO was added to each well and the absorbance was measured at 535 nm using a microplate reader (Bio-Rad 550; Bio-Rad, Hercules, CA, USA).

Western blotting. Cells were collected and cell lysis was performed using RIPA lysis buffer containing protease inhibitor on ice. The extracted protein was quantified by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Then, the total cellular proteins were subjected to 12.5% gradient polyacrylamide gel electrophoresis (e-PAGEL; ATTO, Tokyo, Japan) and transferred to nitrocellulose membranes. The membranes were blocked with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE, USA) for 2 h and then incubated with the respective primary antibody overnight at 4˚C. Following three washes with TBS-T for 10 min, the membranes were incubated with IRDye 680CW-conjugated goat anti-rabbit IgG (1:10,000; LI-COR Biosciences, Lincoln, NE, USA) or IRDye 800CW-conjugated goat anti-mouse IgG (1:10,000; LI-COR Biosciences) for 1 h at room temperature. The western blot images were processed and analyzed using Odyssey infrared imaging system software (LI-COR Biosciences).

We used primary antibodies against the following proteins: E-cadherin (1:500; 180223; Invitrogen, Carlsbad, CA, USA), N-cadherin (1:500; 18022; Invitrogen), vimentin (1:500; sc-6260; Santa Cruz Biotechnology, Dallas, TX, USA), and CK19 (1:1,000; M0888; Dako, Glostrup, Denmark). β-actin (1:10,000, A5441; Sigma Aldrich, St Louis, MO, USA) was used as an internal control.

RT-PCR. Total mRNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and reverse transcription was performed using an AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, Santa Clara, CA, USA). RT-qPCR was performed with a Mx3000P (Agilent Technologies) using a SYBR Green PCR Kit (QIAGEN, Hilden, Germany) in triplicate using specific primers. The primer sequences used to determine expression of the target genes were as follows:
Snail, forward: 5'-TGCAGGACTCTAATCCAAGTTTACC-3' and reverse: 5'-GTGGGATGGCTGCCAGC-3'
Slug, forward: 5'-GGTCAAGAAGCATTTCAAC-3' and reverse: 5'-CTGAGCCACTGTGGTCCTTG-3'
CD44, forward: 5'-AGAAGGTGTGGGCAGAAGAA-3' and reverse: 5'-AAATGCACCATTTCCTGAGA-3'
CD133, forward: 5'-ATGCTCTCAGCTCTCCCGC-3' and reverse: 5'-TTCTGTCTGAGGCTGGCTTG-3'
CK19, forward: 5'-GTCACAGCTGAGCATGAAAG-3' and reverse: 5'-TCACTATCAGCTCGCACATC-3'

The PCR consisted of 95˚C for 10 min followed by 40 cycles of denaturation at 95˚C for 30 sec, annealing for 1 min at 55˚C and a primer extension for 1 min at 72˚C. The comparative CT method was used to quantitate gene expression using *GAPDH* as the internal control.

Statistical analysis. Values on the graphs represent mean±SE. Where indicated, data were subjected to Student's *t*-test and differences were considered significant when *p*-values were less than 0.05

Results

Survival rate of HCC cells after heat treatment. We determined the survival rate after 10 min of heat treatment at 37˚C, 44˚C, 46˚C, 48˚C, or 50˚C (Figure 1A). The survival rate after heat treatment was decreased as the temperature increased. The lethal temperature that killed 50% of HepG2 and HuH7 cells was around 46-48˚C. Similarly, the survival rate after heat treatment at 46˚C for 0, 5, 10, 15, 20, 25, or 30 min was examined (Figure 1B). The survival rate decreased as the duration of treatment increased. Incubation at 46˚C for 10-15 min resulted in 50% HepG2 and HuH7 cell death. Under the same conditions of heat treatment, the survival rate of HepG2 cells tended to be lower than that of HuH7 cells.

Heat shock response of HCC cells. HepG2-L and HuH7-L were exposed to various conditions of heat treatment, and the heat shock response was observed using a reporter system driven by the promoter. HSP70 promoter activity was clearly modulated by both temperature as well as duration of treatment. Baseline luminescence intensity of non-heated HepG2-L cells was 5233 RLU and that of HuH7-L cells was 43342 RLU. The relative level of induction of HSP70 promoter, compared with the non-heated cells, after 10 min heat treatment at 37˚C, 44˚C, 46˚C, 48˚C, and 50˚C was 0.92, 9.7, 35, 24, and 0.37, respectively, in HepG2-L, and 1.1, 4.4, 11, 5.2, and 0.27, respectively, in HuH7-L (Figure 1C). The activity of the HSP70 promoter in HepG2-L and HuH7-L cells after 10 min of heat treatment peaked at 46˚C.

The activity of the HSP70 promoter was also examined following incubation at 46˚C for 5, 10, 15, 20, 25, and 30 min. The peak activity of the HSP70 promoter in both cell lines peaked at 10 min (Figure 1D). At 44˚C, the response of HepG2-L peaked at 25 min and that of HuH7-L peaked at 20 min (Figure 1E). At 48˚C, the response of both cell lines peaked at 10 min and dramatically decreased at 15 min (Figure 1F). A careful examination indicated that the survival rate after heat treatment under severe conditions (high temperature or long duration time) was decreased, which contributed to reduced expression of the HSP70 promoter. Considering the decreasing survival rate after heat treatment, induction of HSP70 promoter per surviving cell was calculated by dividing the response by the survival rate. At 10-min heat treatment, induction of HSP70 promoter per surviving cell peaked at 48˚C (Figure 1G). At 46˚C heat treatment, the response peak per surviving cell was at 20 min (Figure 1H).

Heat treatment reduced the proliferation of HCC cells. The effects of 10 min heat treatment at 37˚C, 46˚C, and 48˚C on the proliferation of HepG2 and HuH7 cells were evaluated (Figure 2A and B). Our data showed that the proliferation rate of heat-treated cells decreased in the order of 48˚C, 46˚C, and 37˚C in both cell lines. The proliferation rate of HepG2 after heat treatment was lower than that of HuH7. However, cell proliferation was not halted completely after severe heat treatment at 48˚C for 10 min, even if the survival rate was reduced by less than half. These data showed that the proliferation rate was decreased after heat treatment.

Phenotypic changes after heat treatment. To determine the influence of heat treatment, HepG2 and HuH7 cells were divided into three groups that underwent heat treatment at 37˚C (as a control), 46˚C, and 48˚C, respectively, for 10 min. After 24 h, phenotypic changes were explored.

To confirm that EMT-like changes had occurred after heat treatment, we assessed the expression of EMT-related transcription factors (snail and slug) by qPCR and EMT markers (E-cadherin, N-cadherin, and vimentin) by western blotting. The levels of snail mRNA in HepG2 and HuH7 treated at 48˚C were significantly higher than those of the control (Figure 3A and B). The levels of slug mRNA tended to be higher after heat treatment (Figure 3C, D). Detection of EMT markers by western blotting demonstrated a reduction in E-cadherin expression, but N-cadherin was up-regulated in HepG2 cells at 48˚C (Figure 3E). Up-regulation of N-cadherin in HuH7 cells treated at 48˚C was observed (Figure 3F). These results indicated that heat treatment could induce EMT-like changes.

As CD44 and CD133 are known CSC markers, we examined whether heat treatment had any effect on the CSC-like properties of HepG2 and HuH7 cells by qPCR.

In HepG2 exposed to 48˚C, the levels of CD44 were significantly higher than those in the control and that of CD133 tended to be higher (Figure 4A, C). The levels of CD44 and CD133 in HuH7 treated with 48˚C were significantly higher than those of the control (Figure 4B, D). CK19 is known as a marker of cholangiocarcinoma and hepatic progenitor cells. CK19 mRNA and CK19 expression after heat treatment were significantly up-regulated compared with the control in both cell lines (Figure 4E, F, G and H). These results indicated that HCC cells could gain CSC-like characteristics by heat treatment.

Evidence of EMT-like changes and CSC-like changes in HCC cells treated at 46˚C were not as obvious as those treated at 48˚C. These data showed that sub-lethal heat treatment could induce phenotypic changes in residual HepG2 and Huh7 cells.

Discussion

We examined the heat shock response of HCC cells after heat treatment for various time periods. Induction of HSP70 promoter became greater as the temperature or the duration of incubation increased; It decreased at longer periods of

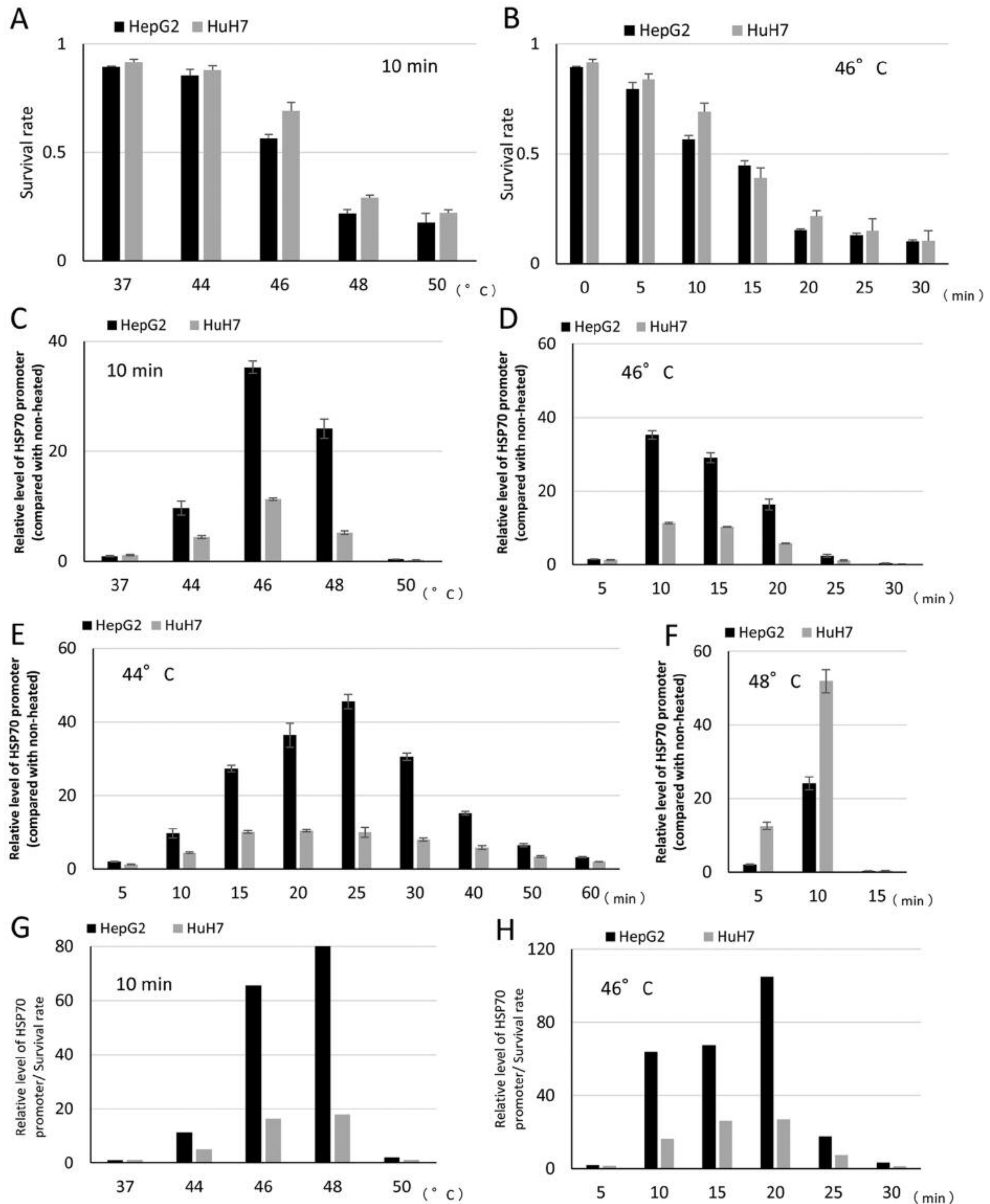

Figure 1. *Survival rate of HepG2 and HuH7 cells after different heat treatment protocols. Ten-min heat treatment at 37˚C, 44˚C, 46˚C, 48˚C, and 50˚C (A). Heat treatment at 46˚C for 0, 5, 10, 15, 20, 25, and 30 min (B). Relative levels of HSP70 promoter induction compared to non-heated control after different heat treatment protocols (C-F). Ten-min heat treatment at 37˚C, 44˚C, 46˚C, 48˚C, and 50˚C (C). Heat treatment at 46˚C for 5, 10, 15, 20, 25, and 30 min (D). Heat treatment at 44˚C for 5, 10, 15, 20, 25, 30, 40, 50, and 60 min (E). Heat treatment at 48˚C for 5, 10 and 15 min (F). Relative levels of HSP70 promoter induction compared to non-heated control per surviving cell after different heat treatment protocols (G, H). Ten-min heat treatment at 37˚C, 44˚C, 46˚C, 48˚C, and 50˚C (G). Heat treatment at 46˚C for 0, 5, 10, 15, 20, 25, and 30 min. Error bars: ±SE of three independent experiments (H).*

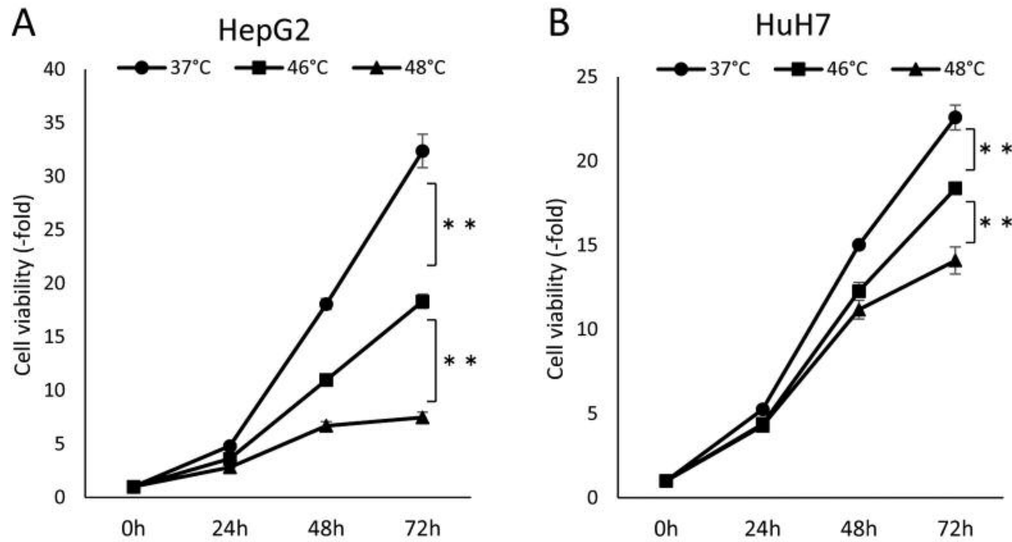

Figure 2. *Proliferation of HCC cells after heat treatment. HepG2 (A) and HuH7 (B) cells were cultured after heat treatment at 37˚C, 46˚C, and 48˚C for 10 min. At 0 h, 24 h, 48 h, and 72 h, viability was evaluated by MTT assay. Error bars: ±SE of three independent experiments. **p<0.01.*

time or higher temperatures because of a decline in the survival of cells. Induction of HSP70 promoter per surviving cell was maximal at sub-lethal heat treatment. At 10-min of heat treatment, the response peaked at 48˚C. Then, we examined the relationship between the heat shock response and the phenotypic changes in residual HCC cells after heat treatment. Sub-lethal heat treatment reduced survival and proliferation rates and induced EMT-like and CSC-like changes that enabled cancer cells to gain more aggressive phenotype.

Local thermal ablation therapies play an important role in the treatment of liver tumors in patients with low disease volume. RFA is a localized thermal treatment technique designed to induce tumor destruction by heating the tumor to a temperature that exceeds 60˚C for around 8-12 min (16, 17). Yamada *et al*. have reported that local recurrence of HCC after RFA showed a higher frequency of portal vein invasion, less tumor differentiation, and worse prognosis compared with HCC without prior RFA (18). Another study has suggested that hyperthermia may play a pivotal role in the rapid proliferation of residual cells *via* the hypoxia inducible factor-1 alpha/vascular endothelial growth factor-A signaling pathway (19).

Heat shock proteins (HSPs) are a family of highly conserved proteins, which are expressed at low levels under normal conditions, but are induced in response to cellular stresses, including heat shock, hypoxia, genotoxic agents, nutrient starvation and overexpression of oncoproteins. The heat shock response is characterized by the preferential synthesis of HSPs, which are molecular chaperones that help proteins to fold correctly during translation and facilitate their transport across membranes. Up-regulation of HSPs is a critical part of heat shock response, which could help cells to cope with the stress condition. The heat shock response is an important biochemical indicator to assess levels of thermal stress (20). The increased expression of HSPs under stress conditions is often transcriptionally regulated by heat shock factor 1 (HSF1). HSF1 is rapidly activated in response to heat stress (21) and HSF1 transcription factor is known to have a strong activation effect on HSP70 promoters (22). HSP70 mediates 14-3-3σ-induced cell migration and tumor development. 14-3-3σ alone or combined with HSP70 are potential prognostic biomarkers for HCC. HSPs are involved in vital mechanisms of cancerous cells, such as cell proliferation, differentiation, invasiveness, neo-angiogenesis, metastasis, and immune system recognition (23). There is growing evidence that HSF1 regulates not only HSPs and stress response, but also many cellular processes including p53, Ras, mitogen-activated protein kinase, cyclic adenosine monophosphate dependent protein kinase A, and mammalian target of rapamycin pathways (24).

In this study, we used the induction of HSP70 promoter as an indicator of the heat shock response of HCC cells to heat treatment. According to our data, after 10-min of heat treatment, induction of HSP70 promoter per well was maximized at 46˚C, and induction of HSP70 promoter per surviving cell was maximized at 48˚C in HepG2 and HuH7 cells. The survival rate after 10-min of heat treatment at 48˚C was less than 50%. Induction of HSP70 promoter of surviving cells was maximized at sub-lethal heat treatment.

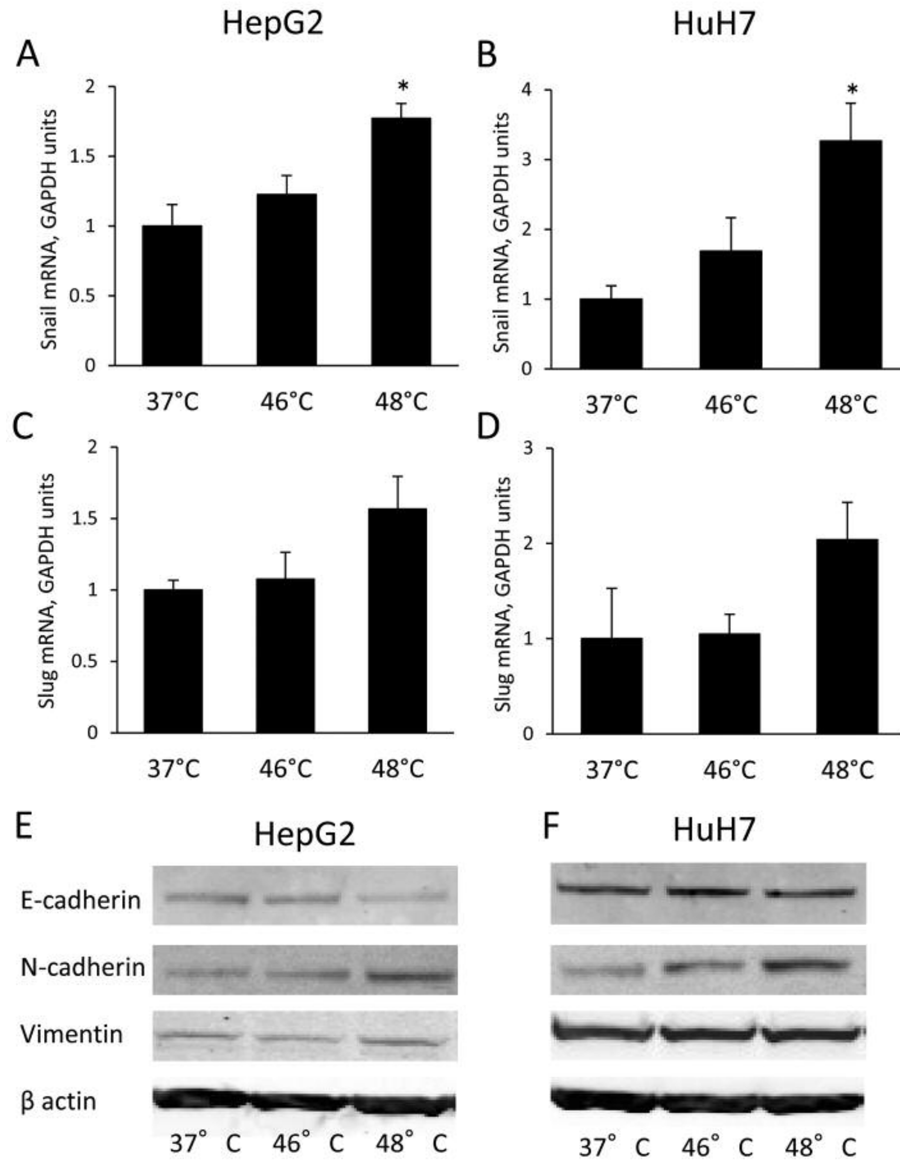

Figure 3. *HCC cells exhibited EMT-like changes after heat treatment. HepG2 cells and HuH7 cells were heat treated at 37˚C, 46˚C, and 48˚C for 10 min. RT-PCR of EMT-related transcription factors Snail (A, B), and Slug (C, D). Western blotting analysis of E-cadherin, N-cadherin, and vimentin (E, F). Error bars: ±SE of three independent experiments. *p<0.05; **p<0.01.*

Recently, it has been shown that acquisition of EMT and induction of CSC-like properties contribute to metastasis (25). Growing evidence has suggested that EMT is a pivotal mechanism of cancer invasion and metastasis, with epithelial cells losing their cell polarity and acquiring the mobility of mesenchymal cells (26). The function of EMT in metastasis involves the down-regulation of epithelial markers, such as E-cadherin, the induction of the expression of mesenchymal markers N-cadherin and vimentin and the up-regulation of transcription factors snail and slug (27). EMT also increases cell migration and invasive characteristics by losing cell–cell adhesion and tight junction, which allows tumor cells to become more easily separated from the original site (28). We showed that heat treatment decreased expression of membranous E-cadherin, up-regulated N-cadherin, and up-regulated snail and slug, which regulate EMT. These findings indicate that sub-lethal heat treatment induces EMT-like change in HCC cells.

CSCs, also known as cancer-initiating cells, are defined as a subpopulation of cells that possess the properties of self-renewal, differentiation, and tumorigenesis (29). CSCs are closely associated not only with carcinogenesis but also with

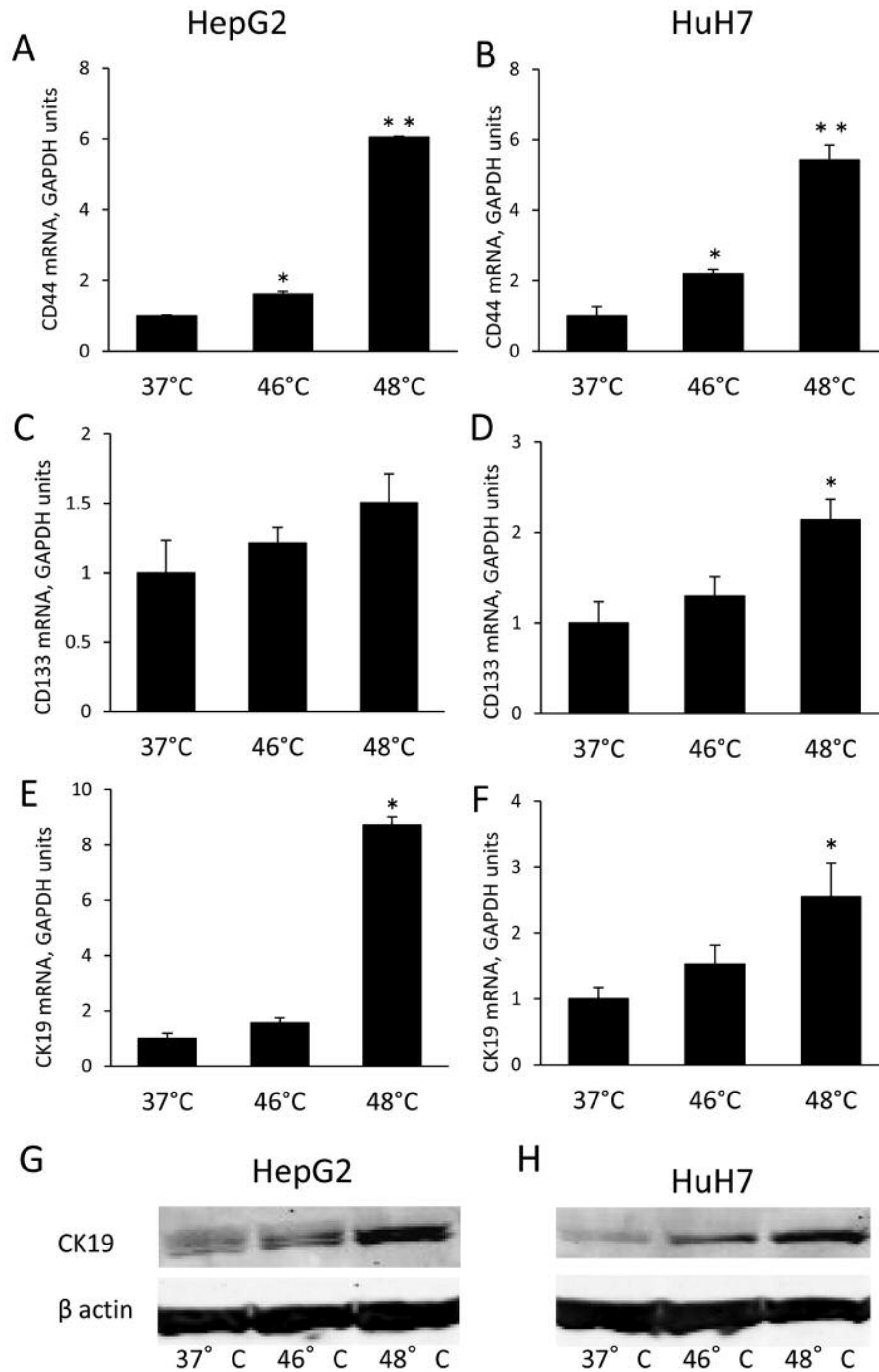

Figure 4. *HCC cells exhibited CSC-like changes after heat treatment. HepG2 and HuH7 cells were heat treated at 37˚C, 46˚C, and 48˚C for 10 min. RT-PCR analysis of CD44 (A, B), CD133 (C, D), and CK19 (E, F) expression. Western blotting analysis of CK19 (G, H). Error bars: ±SE of three independent experiments. *p<0.05; **p<0.01.*

tumor recurrence and metastasis (30). CD44 and CD133 are known as markers of CSCs (31). Increased expression of CD44 and CD133 indicate poor prognosis in HCC (14) suggesting that HCC cells acquire aggressive, metastatic and infiltrative phenotypes through cancer stemness. Wu *et al*. have reported that, in some cases, primary HCC express biliary differentiation (32). CK19 is known as a biliary differentiation marker and is expressed in hepatic progenitor cells that possess potential to

differentiate toward the hepatocytic or biliary phenotype (29, 33). CK19 is also expressed in cholangiocarcinoma, cholangiolocellular carcinoma and combined hepatocellular and cholangiocarcinoma (34). CK19 positivity in HCC strongly correlates with increased malignant properties, and is a predictive factor for prognosis (35, 36). Our data showed that the expression of CD44, CD133 and CK19 were increased after heat treatment.

These phenotypic changes were significant after heat treatment at 48˚C for 10 min. At this temperature, survival and proliferation rates were decreased, but induction of HSP70 promoter per surviving cell was high. These targets may be involved in the regulation of EMT-like and CSC-like phenotypes and lead HCC cells to become more aggressive.

RFA is one of the effective and powerful treatment choices for the local control of HCC, particularly when patient's liver functional reserve is insufficient and does not permit hepatic surgical resection. However, sub-lethal heat treatment could occur at the peripheral zone of any incomplete ablation and at the tissue–vessel interface where flowing blood thermally protects tissue and causes a heat-sink (37). After incomplete ablation, surviving cells might gain aggressive phenotypic changes. However, our data do not diminish the importance of RFA. These findings suggest that complete ablation, sufficiently high temperatures, and secure wide therapeutic margins are required. Furthermore, these findings could be a potential mechanism associated with the aggressiveness of any surviving tumor cells after RFA. However, we investigated only the thermal effects *in vitro*. Further studies are required to clarify the malignant potential of residual HCC cells after incomplete RFA.

In conclusion, sub-lethal heat treatment in HCC promotes EMT-like changes in cancer cells and acquisition of a stem cell phenotype. These phenotypic changes may render HCC cells more aggressive.

Conflicts of Interest

The Authors report no proprietary or commercial interest in any product mentioned or concept sed in this article, and this work was not supported by any specific grants.

Author's Contributions

Authors' contributions Ryosuke Zaimoku designed the study, and wrote the initial draft of the manuscript. Tomoharu Miyashita contributed to analysis and interpretation of data, and assisted in the preparation of the manuscript. All other Authors have contributed to data collection and interpretation, and critically reviewed the manuscript. All Authors approved the final version of the manuscript, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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