Additional Malignant Transformation of a Hepatocellular Carcinoma Cell Line by Heat Treatment Can Occur Through Epithelial-Mesenchymal Transition without Acquisition of Heat Resistance

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Abstract

Background and Aim: Radiofrequency ablation (RFA) is a thermoablative technique used to kill tumor cells by generating heat and coagulative necrosis. Although it is widely used for hepatocellular carcinoma (HCC), there are some case reports in regard to local recurrences of HCC with aggressive phenotypes. We previously reported that transformation of HCC cells could be induced by a heat treatment (HT) of the type associated with RFA. However, its mechanism is still unclear.

The aim of our current study was to determine whether this HT-induced transformation occurs through epithelial-mesenchymal transition (EMT).

Methods: We established 3 HepG2 sub-clones (Clones A, B, and C), which were cloned from a colony that survived a single HT, and 3 HepG2 sub-lines (5HA, 5HB, and 5HC), which survived 5 rounds of HTs. We examined their morphology, proliferation speeds, heat resistances, and gene expressions, as well as the tumorigenicity of Clone C.

Results: All sub-clones and sub-lines of HepG2 used in this study grew in a semi-adherent manner. They proliferated 30–50% faster than the parental line under anchorage-dependent conditions, and 70–100% faster under anchorage-independent conditions. All HepG2 sub-clones expressed lower levels of E-cadherin and higher levels of alpha smooth muscle actin and vimentin, and sub-lines showed similar tendencies, suggesting the occurrence of EMT. Interestingly, none of them demonstrated heat resistance. When Clone C cells were inoculated into nude mice, they generated tumors in 8 mice out of 9 mice within 4 weeks, whereas the parental HepG2 cells generated tumors in 3 mice out of 9 mice, indicating the acquired tumorigenic potential of the sub-clone.

Conclusion: These findings suggest that HT can induce additional malignant transformation of HCC cells through EMT, without the acquisition of heat resistance.

Key words
heat-treatment, hepatocellular carcinoma, epithelial-mesenchymal transition

Background

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers1–4 and the third most frequent cause of cancer-related death worldwide.5–7 One of the clinical characteristics of HCC is that it most often develops inpatients with chronic liver disease.8–11 This information is beneficial from the point...
of view of early detection, physicians can identify a group of patients at high-risk for HCC and perform screening examinations on a regular and systematic basis. Unfortunately, the recurrence rate of HCC after curative treatment is relatively high due to background chronic liver disease. To determine a treatment strategy against HCC, we need to evaluate not only the tumor factors, but also the patient’s background liver function. Considering the high rate of recurrence and the need to prioritize the preservation of liver function, less invasive therapy is preferable especially for early-stage HCC.

Radiofrequency ablation therapy (RFA) is frequently used as a local treatment in HCC, and its efficacy has been reported worldwide. It is an electrosurgical technique that uses a high-frequency alternating current over a short period of time to heat tissues to the point of thermal coagulation. Because of its ability to achieve tumor necrosis, its minimal damage to the functioning liver tissue, and easy repeatability in case of recurrence and/or new tumor growth, RFA has become widely accepted for the treatment of patients with HCC.

There are case reports, however, which indicate that residual HCC after RFA treatment may exhibit an aggressive phenotype with an unfavorable prognosis. These reports indicate the possibility that the heat stress induced by RFA could induce the additional malignant transformation of HCC cells. Not only RFA, but also other anticancer therapies may accelerate the so-called sarcomatous change of HCC. Some reports suggest that sarcomatous change is closely related to epithelial-mesenchymal transition (EMT).

From these clinical observations, we hypothesized that RFA may be involved in this type of transformation. We previously reported that even a single heat treatment (HT) in vitro, which mimics insufficient RFA, can induce additional transformation of an HCC cell line. This was the first report to indicate the potential for malignant transformation through HT. Ke et al reported a similar phenomenon with an in vivo experiment using rabbits. Recently, using the experimental system that we developed, Kong et al generated an HCC sub-line which obtained tumorigenicity and the ability to promote angiogenesis, indicating the reproducibility of the additional malignant transformation induced by heat treatments. However, the mechanism of this transformation is still unclear.

The aim of this study was to prove that the additional malignant transformation induced by HT occurs through EMT.

Materials and Methods

Cell culture
A human hepatoma cell line (HepG2) was used in this study, because we had successfully obtained a transformed HepG2 sub-line by HT in our previous study. This cell line was maintained as a monolayer culture in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 2 × 10^4 U/L penicillin G, and 200 mg/L streptomycin sulfate. All cell lines were incubated at 37°C in a humidified incubator under a flow of 5% CO₂ and 95% air.

HT and generation of sub-clones and sub-lines
Cells were exposed to hyperthermic stress as previously described. Approximately 1 × 10^3 cells were seeded into each well, in 96-well plates, with 50 µL of DMEM. After 24 h of incubation, they were exposed to heat stress: HT was carried out by submerging the plates in a water bath set to the desired temperature for 10 minutes. Immediately after the hyperthermic treatment, 100 µL of fresh culture medium was added into each well, and cells were maintained in the incubator at 37°C. When the surviving cells became 60–80% confluent, they were re-plated onto a 6-well plate and a 10-cm dish. To isolate clones, we re-plated cells from each of 6 wells onto 5 dishes of 15 cm. Single colonies were isolated using cloning cylinders.

Cell proliferation assay
To estimate the proliferation speed of cells, we used WST-8 cell proliferation assay using Tetracolor One assay solution (Seikagaku Co., Tokyo, Japan). All of the WST-8 assays were performed in octuplicate, using 96-well plates for anchorage-dependent growth, or Ultra-Low Attachment 96-well plates (Sigma-Aldrich Co., St. Louis, MO, USA) for anchorage-independent growth. The data indicated in the figures are the average of 3 independent experiments, and error bars represent standard error (SE).

Real-time quantitative polymerase chain reaction (q-PCR)
Total RNA was isolated from cells using the RNaseasy Mini Kit (QIAGEN, Valencia, CA, USA) and was quantitated by using spectrometry at 260 nm. Complementary DNA (cDNA) was synthe-
sized from 1 µg of total RNA, with the PrimeScript RT Reagent Kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer’s instructions. The cDNA samples were subjected to PCR amplification with specific primers, indicated below. The PCR reaction mixture was augmented with SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan) and distilled water to a final volume of 20 µL.

We performed q-PCR using the PCR primers indicated below on a real-time PCR cycler (ABI PRISM 7500 Fast Real-Time PCR system, Applied Biosystems, Foster City, CA, USA). All experiments were performed in triplicate and normalized to GAPDH messenger RNA (mRNA) expression. Fluorescent signals were analyzed during each of up to 40 cycles. Each cycle consisted of denaturation (95°C, 3 s), annealing, and extension (60°C, 15 s). Relative quantitation was calculated using the comparative threshold cycle method (CT, as described in the User Bulletin, ABI PRISM 7500 Fast Real-Time PCR system). CT indicated the fractional cycle number at which the amplified gene equaled a fixed threshold within the linear phase of amplification. The median CT value of triplicate measurements was used to calculate ΔCT. ΔCT of each gene for each sample was compared with the ΔCT of GAPDH for the same sample, expressed as ΔΔCT. Relative quantification was depicted as fold-change expression for each gene, compared with GAPDH mRNA, using the formula \(2^{-\Delta\Delta CT}\). Denaturing curves were used to confirm homogeneity of the DNA products.

The forward (F) and reverse (R) PCR primers used in this study were as follows:

- GAPDH: (F) 5'-GAAAGTGAAAGTCGGAGTCA-3' and (R) 5'-GAAGATGGTGATGGGATTTC-3',
- E-cadherin: (F) 5'-CCAAAAATGTGATGAGGGTG-3', and (R) 5'-GGGGGTTAAGTTGAGGGTA-3',
- Alpha smooth muscle actin (α-SMA): (F) 5'-ACCCACAATGTCCCATCTA-3', and (R) 5'-GAAGGAATAGCCACCGTTCAG-3',
- Vimentin: (F) 5'-CCGACACTCTCAAGATTTAGA-3', and (R) 5'-CAAAGATTATTTGAAGCAGAACC-3'

Animal experiments

Six-week-old male nude mice (Crlj:CD1-Foxn1/nu, Charles River Laboratories International, Inc, Wilmington, MA, USA) were used in this experiment. We suspended cells at a concentration of 5 × 10^7 cells/mL in DMEM and subcutaneously injected the cells into 9 mice with 200 µL of suspension. We measured tumor sizes once a week. This study was carried out in strict accordance with the recommendations in the guide for the Care and Use of Laboratory Animals of Experimental Animals, St. Marianna University School of Medicine. (approval #: 0902013)

Statistical analysis

Values on the graphs represent means ± SE. Where indicated, data were subjected to Student’s t-test, and differences were determined to be significant when the p value was less than 0.05.

Results

Generation of sub-clones and sub-lines that survived HT(s)

In the previous study, to obtain a transformed sub-line, we examined a total of 60 sub-lines, originating from 3 HCC cell lines. In this study, to emphasize the effect of HT, we set the temperature higher and cultured cells longer than in the previous study. Here we performed HT at 49°C for 10 min, and cells were subsequently cultured for 7 weeks, while the treatment temperature was 47°C and the culture duration was 2 weeks in the previous study. Reproliferation of surviving cells occurred in 23 out of 96 wells. The morphologies of the colonies that survived HT were different from those of the original colonies (Figure 1a); they were caviar-like, flat, and bubble-containing morphologies, as shown in Figure 1b, c, and d. Only 1 well showed reproliferation within 5 weeks of HT; the cells in this well had a spindle-like appearance, shown in Figure 1e. Because the morphological difference was quite obvious, and because these cells started to proliferate faster than the other cells, we decided to establish clones from this colony. We performed single-cell cloning and expanded 3 sub-clones. These sub-clones proliferated in a semi-floating manner as shown in Figure 1f. These cells could be continuously cultured without trypsinization, and the morphology of the adherent cells reverted to the original (Figure 1g). These sub-clones were named Clones A, B, and C.

To estimate the reproducibility of the emergence of such cells through HTs, we performed 5 rounds of weekly HTs: twice at 49°C and 3 times at 50°C. From 288 starting wells, surviving cells were observed in 24. Three wells showed similar morphology to Clones A, B, and C, and we selected and expanded these wells (Figure 1h). These sub-lines were named 5HA, 5HB, and 5HC.
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Proliferation speed

We estimated the proliferation speeds of the sub-clones, and sub-lines. Clones A, B, and C proliferated significantly faster than the parental HepG2 cells in adherent conditions (Figure 2a), as did sub-lines 5HA, 5HB, and 5HC (Figure 2b). Because the sub-clones and sub-lines proliferated in a semi-adherent manner, we next examined their proliferation speeds under anchorage independent conditions. On day 3, the cell numbers of all sub-clones and sub-lines (Figure 2c) were significantly larger than the number of parental HepG2 cells.

Heat sensitivity

As these sub-clones and sub-lines were generated by HTs, it was possible that they had developed a tolerance against heat. We therefore assessed their heat tolerance to their parental HepG2 cells. The number of surviving cells 72 h after HT at 46˚C, 47˚C, and 48˚C was estimated by the WST-8 assay. None of the sub-clones and sub-lines showed a significant difference from the HepG2 cells. (Figure 3)

Gene expression analysis

Because the sub-clones and sub-lines proliferated in a semi-adherent manner, we estimated the expression of E-cadherin mRNA by using q-PCR. Expression levels of E-cadherin mRNA in the 3 sub-clones were approximately 30–50% of HepG2 cells (Figure 4a). On the other hand, α-SMA and Vimentin expressions in the sub-clones were 2.5–5 and 3–5 times higher than that in HepG2, respectively (Figure 4b, c). On the other hand, only E-cadherin expression in 5HA and α-SMA expression in 5HC showed a significant difference in comparison to those in the parental cells, although similar tendencies were observed in the 3 sub-lines.

Tumorigenicity in nude mice

Finally, to confirm that the transformation induced by HT was related to malignant potential, we assessed the tumorigenicity of Clone C compared...
with that of HepG2 cells. We chose only one clone, since similar results were expected if we had used other sub-clones. When $1 \times 10^7$ cells each of Clone C and HepG2 were subcutaneously injected into nude mice, Clone C generated tumors in 8 out of 9 mice within 4 weeks, whereas parental HepG2 cells generated tumors in only 3 mice out of 9 mice (Figure 5a). On average, the Clone C tumors were significantly larger than the parental HepG2 tumors (Figure 5b). This result indicates that Clone C acquired tumorigenicity.

**Discussion**

In this study, we showed that HT could reproducibly lead to additional malignant transformation. Successfully generated sub-clones and sub-lines showed similar morphology to each other and demonstrated faster proliferation than the parental HepG2 cells. If these lines existed before the heat treatment, the cells with such a phenotype should have become the majority. We therefore concluded that these data indicated an acquired proliferation capacity of sub-clones and sub-lines, suggesting that additional transformation occurred through HT. The mRNA expression patterns may suggest that their transformation occurred through EMT. Although we saw some differences in gene expression between sub-clones and sub-lines (Figure 4a–c), these differences can be explained by the difference of the methods to generate sub-clones and sub-lines. Concretely speaking, 3 sub-clones originated from a single cell, while 3 sub-lines did not. Collecting all these data, we conclude EMT might occur through HT in HepG2 cells. Moreover, one of the sub-clones showed the acquirement of tumorigenicity, proving the occurrence of additional malignant transformation. These data support the possibility that EMT of HCC cells induced by insufficient RFA could be a cause of local recurrence with aggressive phenotype and unfavorable prognosis, as our experimental system mimics the microenvironment of HCC cells during RFA.

However, not all surviving colonies showed this
Figure 3. Numbers of surviving cells 72 h after HT at each temperature.
   a) Parent, b) Clone A, c) Clone B, d) Clone C, e) 5HA, f) 5HB, g) 5HC

Figure 4. Expression levels of EMT marker genes in sub-clones and sub-lines.
   Expression levels of a) E-cadherin, b) α-SMA, c) Vimentin
   The error bar represents the SE from 3 independent experiments. *p<0.05 vs. Parent
type of transformation. As shown in Figure 1, a variety of morphologically different colonies was observed after HT. Among the cells in 24 wells, which survived 5 rounds of weekly HTs, the cells in the residual 21 wells did not show spindle-like morphological change. In addition, the phenotypes of the sub-line obtained in our previous study were different in several ways. Morphological change was not observed, and the sub-line in our previous study showed the marked heat tolerance. Furthermore, the mechanism to acquire heat resistance through HT still remains to be elucidated. Recently, Kong et al reported the promotion of angiogenesis because of insufficient RFA, with this phenomenon occurring through HIF-1α/VEGFA stimulation. This report is proof of the reproducibility of our previous experimental system, and provides another explanation of the rapid progression of locally recurrent HCC after RFA. These findings suggest that HT as a survival stressor can elicit diverse responses, including EMT, which does not necessarily require the acquisition of heat resistance. Because HT is not a physiological but a physical survival stressor, it may randomly cause further genetic or epigenetic changes in cancer cells that have, by definition, inherently acquired genetic and/or epigenetic alterations. Our data show that at least some of the reactions are irreversible.

Among the different types of colonies produced, we focused on the spindle-like cells, since we had decided to investigate the possibility of EMT involvement in the transformation induced by HT. The sub-clones showed reduced expression of E-cadherin and enhanced expression of vimentin and α-SMA, and the sub-lines showed similar tendencies, suggesting the occurrence of EMT. This finding suggests that EMT can reproducibly occur through HT and that it may be one of the mechanisms of the malignant transformation induced by RFA.

It has recently been suggested that cancer stem cells are involved in the EMT of HCC and that EMT is related to tumor progression. Involvement of cancer stem cells in EMT has also been reported elsewhere. Interestingly, we found that all sub-clones and sub-lines strongly expressed CD133, a putative marker of HCC stem cells. Pyrosequencing analysis revealed that this expression was regulated epigenetically; namely, the methylation level of

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**Figure 5.** Acquired tumorigenicity of Clone C in nude mice.  
Each arm contains nine animals. **a)** The number of nude mice with tumor formation. **b)** Weekly assessment of tumor size. Bar graph shows the result of statistical analysis at day 28.  
*p<0.05 vs. Parent*
GC sequence in the promoter lesion of CD133 was unmethylated in Clone C (data not shown). There may be 2 possibilities to explain this result. First, CD133-positive cancer stem cells are directly involved in the EMT of HCC. In this scenario, heat stress as a survival stressor might induce the inappropriate differentiation of cancer stem cells. Another possibility is that CD133 expression has nothing to do with EMT, but is a result of a random epigenetic change induced by HT. The involvement of epigenetic change and cancer stem cells in the additional malignant transformation induced by HT still needs elucidation.

From a clinical point of view, the frequency of this additional malignant transformation through EMT is important; this information could affect the treatment strategy for local recurrences after RFA. In this study, we observed transformation in only 1 well out of 23 wells with cells surviving a single HT, and in 3 wells out of 24 wells with cells surviving 5 rounds of HTs. These findings suggest that the additional malignant transformation through EMT induced by HT is a relatively rare event. Additionally, even if tumor cells acquire rapid proliferative potential, the HCC cells do not necessarily acquire heat resistance. In the clinical setting, once incomplete ablation is suspected, physicians should plan an adequate treatment for local recurrence.³⁴) Repeat RFA does not have to be excluded from the possible treatment strategies because tumor cells rarely transform, as is indicated in this study. This idea is validated by clinical literature, which shows the high repeatability of RFA.³⁵³⁶) However, from a view-point of local control, surgery may be a good option for treating a locally recurrent tumor, particularly when additional transformation is suspected.

Conclusion

Our findings suggest that HT, which mimics insufficient RFA, can induce additional malignant transformation of surviving HCC cells through EMT. This transformation does not necessarily acquire heat resistance.

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