SPANDIDOS PUBLICATIONS

Effect of hyperthermia on invasion ability and TGF-ß$_1$ expression of breast carcinoma MCF-7 cells

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Abstract. The present study aimed to investigate heating-induced alterations of breast cancer cell invasion abilities and the potential mechanisms associated with TGF-ß$_1$ expression. MCF-7 cells were heated at 43, 45, 47 and 37˚C for 30 min. *In vitro* cell invasion ability was evaluated by matrigel invasion assay. The activity of matrix metalloproteinase (MMP)-2/9 was investigated by gelatin zymographic assays. Expression of vascular endothelial growth factor (VEGF) and transforming growth factor-ß$_1$ (TGF-ß$_1$) was investigated by immunocytochemistry and RT-PCR. Apoptosis was analysed by flow-cytometry. The invasive potential of MCF-7 cells was reduced by heating, and MMP-2/9 secretion and enzymatic activity were suppressed. Furthermore, VEGF and TGF-ß$_1$ mRNA and proteins were suppressed by hyperthermia. These results suggest that down-regulation of the expression of TGF-ß$_1$, EGF and MMPs by hyperthermia probably accounts for the inhibition of the invasive abilities of MCF-7 cells.

Introduction

Hyperthermia is a promising approach to cancer therapy because it not only kills cancer cells directly, but also can alter the character of tumor cells with high metastatic potential. The experimental study *in vivo* by Nagashima *et al* suggested that local hyperthermia (heating at 43˚C for 40 min) inhibited lymph node metastasis of hamsters oral squamous cell carcinoma when the primary tumor responded histologically to hyperthermia treatment (1). There are also reports on *in vitro* research to explain the mechanism. It was reported that heat shock at 42˚C suppressed the production of Membrane-type 1 MMP (MT1-MMP), which in turn inhibited MMP-2 activation and increased release of tissue inhibitor of MMP-2 from cell surface (2). A further study has demonstrated that a transient increase in intracellular cAMP was a critical signal for heat shock to induce tumor specific-suppression of MT1-MMP production and proMMP-2 activation in HT-1080 cells after heat shock at 42˚C for 3 h (3). Sawaji *et al* have reported that when HT-1080 cells were treated with heat shock at 42˚C for 4 h and maintained at 37˚C for another 24 h, the gene expression of all vascular endothelial growth factor variants were suppressed (4).

Most of the investigations have focused on the MMPs and VEGF under hyperthermia. Moreover, TGF-ß$_1$ also modulates the processes of cells invasion and micro-environment modification that cancer cells may exploit to their advantages. *In vitro* studies have revealed that several key angiogenic mediators such as VEGF are direct targets of the TGF-ß$_1$ signal pathway (5-7) and TGF-ß$_1$ can induce a pro-angiogenic environment and stimulate angiogenesis. Additionally, TGF-ß$_1$ can regulate the expression, secretion and activity of MMP-2 and MMP-9, and down-regulate the expression of the protease inhibitor TIMP in tumor and endothelial cells (8,9). By these metalloproteinase activities, TGF-ß$_1$ can enhance the migratory and invasive properties of tumor and endothelial cells during angiogenesis. However there are few reports in the literatures on TGF-ß$_1$ expression of carcinoma cells treated with hyperthermia. Thereby, we need a fuller understanding of the alteration of TGF-ß$_1$ in cancer by hyperthermia, which will guide us toward the goal of optimizing the use of these agents together with hyperthermia in the clinic.

MCF-7 cells, a human breast adenocarcinoma cell line, that can form tumors when injected into athymic nude mice and be able to metastasis to lungs, liver and spleen (10), were used as a model in this heat shock study. This study probed the invasive potentials and expression of TGF-ß$_1$, VEGF and MMP-2/9 in cultured MCF-7 cells by heating at 43, 45, 47 and 37˚C for 30 min. The results of these experiments were

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used to explain the mechanism of *in vitro* hyperthermia effect on invasiveness and metastases of carcinoma.

Materials and methods

Cell culture. MCF-7 cells were obtained from the National Centre for Cell Sciences and were grown and maintained in RPMI-1640, containing 10% FBS in a 5% CO_2 incubator at 37˚C.

Cell treatment. Exponential growing cells were sealed using parafilm in cell culture flasks (1x10^6 cells/flask) and immersed in a thermostated water bath at the indicated temperature (43, 45 and 47˚C) for 30 min and the control group was treated at 37˚C for 30 min. The temperature of the medium increased quickly and reached the setted temperatures within 3 min. The temperature in the medium was monitored with the fiber optic thermometer probe (FX-9020; Anritsu Meter Co., Tokyo). After heating, the cells were immediately incubated at 37˚C in 5% CO_2.

Matrigel invasion and metastases assay. The invasion activity of MCF-7 cells after heating at different temperature was assayed in a transwell cell culture chamber (6.5 mm, 8 μm Costar, Corning). Polyvinylpyrrolidone-free polycarbonate filters were smeared with an 8.0 μg fibronectin (GenScript Cat. No. RP10840) in a volume of 10 μl serum-free RPMI-1640 on the reverse side, and dried at room temperature. Matrigel (containing laminin, collagen type IV, heparan sulfate proteoglycan and entactin) (BD Biosciences Cat. No. 356230), was diluted to 500 μg/ml with cold phosphate-buffered saline (PBS), applied to the upper surface of the filter (5 μg/filter), and dried at room temperature. MCF-7 cells which were heated in a water bath for 30 min and then incubated for 2 h at 37˚C in 5% CO_2 were harvested with 1 mM EDTA in PBS, washed twice and resuspended to give a final concentration of 2.0x10^6/ ml in serum-free RPMI-1640 with 0.1% bovine serum albumin (BSA). Cell suspensions (100 μl) were added to the upper compartment and the filter chamber was incubated for 24 h at 37˚C in a 5% CO_2 atmosphere. The cells on the upper surface of the filters were removed by wiping them with a cotton swab. The filters were then stained with crystal violet for 30 min. The cells that had invaded through matrigel and reached to the reverse side were counted under a microscope in five pre-determined fields at a magnification of x400. Each assay was performed in triplicate.

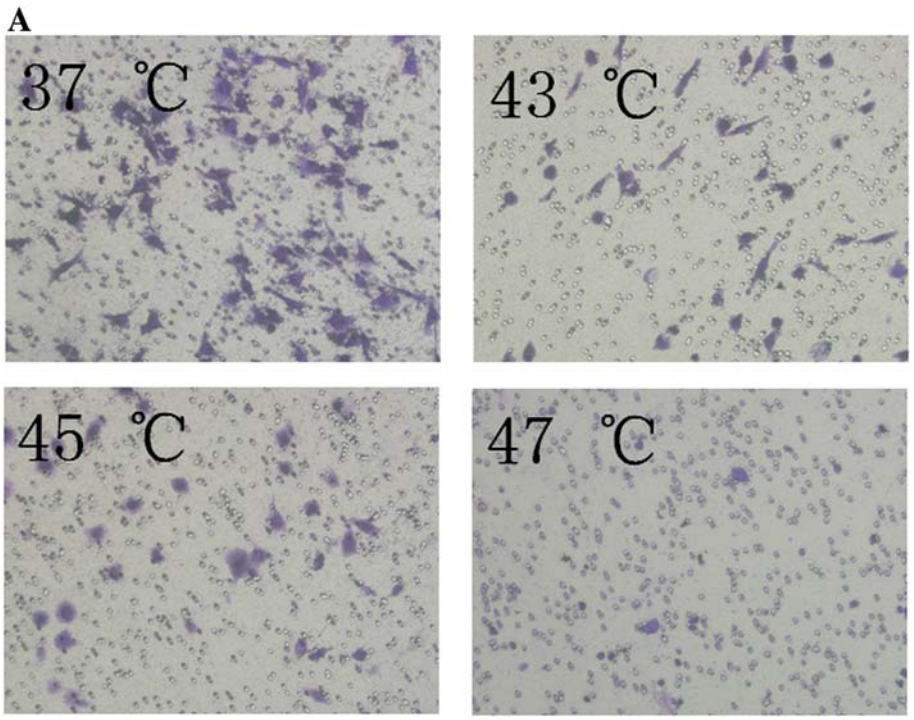
Gelatin zymography. MCF-7 cells were heated in a water bath for 30 min as has been noted and then incubated for 24 h with serum-free RPMI-1640 at 37˚C in 5% CO_2. The culture supernatant of them was collected by centrifugation and then concentrated by lyophilization. The extract was subjected to zymography on 7.5% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) co-polymerized with 0.1% gelatin. Gel was washed in 2.5% Triton-X-100 for 30 min to remove SDS and was then incubated overnight in a reaction buffer (50 mM Tris-HCl pH 7, 4.5 mM $CaCl_2$, 0.2 M NaCl). After incubation, the gel was stained with 0.5% coomassie blue containing 30% methanol and 10% glacial acetic acid. The bands were visualized by destaining the gel with the solution containing 30% methanol and 10% glacial acetic acid. The experiments were repeated three times with similar conditions and one experiment was selected for representation.

Immunocytochemistry. MCF-7 cells grown on coverslips were treated by hyperthermia at 43, 45 and 47˚C, and the control group was treated at 37˚C for 30 min. After 24 h, cells were fixed in 4% paraformaldehyde/PBS for 30 min, permeabilized in 0.5% Triton X-20/PBS for 3 min and blocked with 50 μl normal goat serum (ZSGB-Bio Beijing, China) for 10 min at room temperature. The cells were incubated overnight at 4˚C with anti-TGF-ß$_1$ or anti-VEGF antibodies (Santa Cruz Biotechnology, Inc.) respectively at a 1:50 dilution in PBS. After three 10-min washes with PBS at room temperature, the cells were incubated for 40 min at room temperature with Polymerized HRP-Anti-rabbit IgG (ZSGB-Bio Beijing), then washed three times in the dark. Cytoplasm and cell membrane were stained with 3,3'-diamino-benzidine tetrahydrochloride bis-benzimide (1:20, ZSGB-Bio) for 5 min at room temperature, and the cells embedded in a gummy mounting for further analysis. While cells were being observed, pictures were taken using a light microscope (Olympus BX51, Tokyo, Japan).

RT-PCR analysis. The expression of TGF-ß$_1$ and VEGF mRNA was analyzed by semi-quantitative RT-PCR. MCF-7 cells were heated by water bath for 30 min as has been noted and then incubated for 24 h. Total RNA was extracted from those cells using TRIzol reagent (Invitrogen, USA), respectively and 5 μg RNA was used to synthesize cDNA using RT-PCR Kit (M-MLV) (Keygen, KGA1305) following the manufacturer's protocols. The cDNA was used to amplify the TGF-ß$_1$ and VEGF mRNA fragment, while the house-keeping gene ß-actin was also amplified as an internal standard. The corresponding primer sequences were as follows: TGF-ß$_1$ forward: 5'-GGCCAGATCCTGTCCAAGC-3', reverse: 5'-GTGGGTT TCCACCATTAGCAC-3'; VEGF forward: 5'-CAACATCA CCATGCAGATTATGC-3', reverse: 5'-GCTTTCGTTTTT GCCCCTTTC-3'; The cycling program was performed as follows: 1 cycle of 94˚C for 5 min; 33 cycles of 94˚C for 30 sec, 54˚C for 45 sec, 72˚C for 60 sec; followed by a final elongation step of 72˚C for 10 min. Then RT-PCR products were electrophoresed through a 1.5% agarose gel with ethidium bromide. The experiments were repeated three times with similar conditions and one experiment was selected for representation.

Cell apoptosis detection. MCF-7 cells were heated by water bath for 30 min as has been noted and then incubated for 24 h. Then the cells were collected for cell apoptosis detection. The Annexin V-FITC apoptosis detection kit (Keygen, KGA107) was used according to the manufacturer's protocol. The analysis was performed using a FACSCalibur flow cytometer. Approximately 10,000 cells were evaluated for each sample. The experiment was repeated three times.

Statistical analysis. Statistical analysis was performed using SPSS (version 13.0). Data were expressed as the mean ± SD for n replicates as indicated in figure legends. One-way



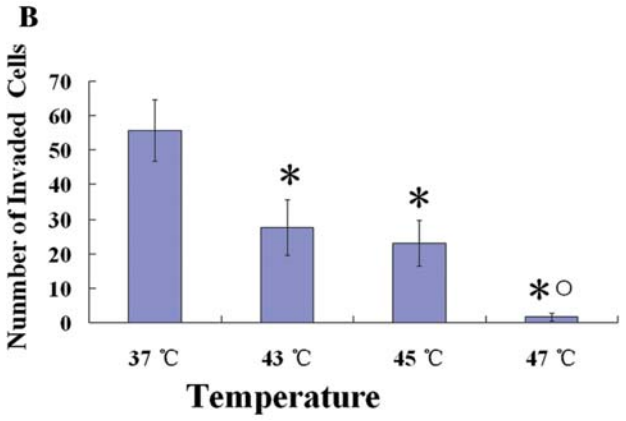


Figure 1A and B. Invasive potentials of MCF-7 cells after hyperthermia. (*) P<0.05 compared with control; (○) P<0.05 compared with the 45˚C group (n=5).

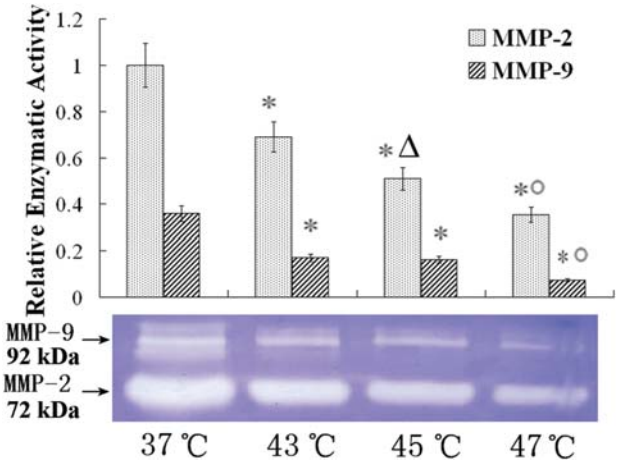


Figure 2. Gelatin zymographic analysis of MMP-2 and MMP-9. (*) P<0.05 compared with control; (Δ) P<0.05 compared with the 43˚C group; (○) P<0.05 compared with the 45˚C group (n=3).

analysis of variance followed by an SNK test was used to assess significant differences between the control and experimental groups.

Results

Hyperthermia inhibits invasive potential of MCF-7. We examined changes in the invasive and metastases potentials of MCF-7 cells after hyperthermia treatment using matrigel invasion assay. Representative pictures of matrigel invasion assay showed a decrease in the number of invading MCF-7 cells after hyperthermia (x400) (Fig. 1A). Column data showed that hyperthermia decreased the invasiveness of MCF-7 in a temperature-dependent manner (Fig. 1B). Compared with control group (37˚C), MCF-7 cells heated at 43, 45 and 47˚C showed significantly lower numbers of invaded cells.

Down-regulation of MMP-2 and MMP-9 activity. In order to see whether the down-regulation of MMP-2 and MMP-9 in MCF-7 cells could be induced by hyperthermia, we examined the effect of hyperthermia treatment at different temperature points in secretion and activity of MMP-2 and MMP-9 by gelatin zymographic analysis. As shown in Fig. 2, white bands were areas degraded by MMP-2 and MMP-9. The corresponding picture represented the quantitative analysis of the band intensities using a contour tool by Quantity One-4.6.2 (Basic) which clearly showed that hyperthermia treatment caused significant inhibition on secretion and activity of MMP-2 and MMP-9 in MCF-7 (P<0.05) and the decrease appeared to be temperature-dependent.

Down-regulation of TGF-β_1 and VEGF protein expression. Immunocytochemistry was performed to see whether the protein expression of TGF-β_1 and VEGF in MCF-7 cells

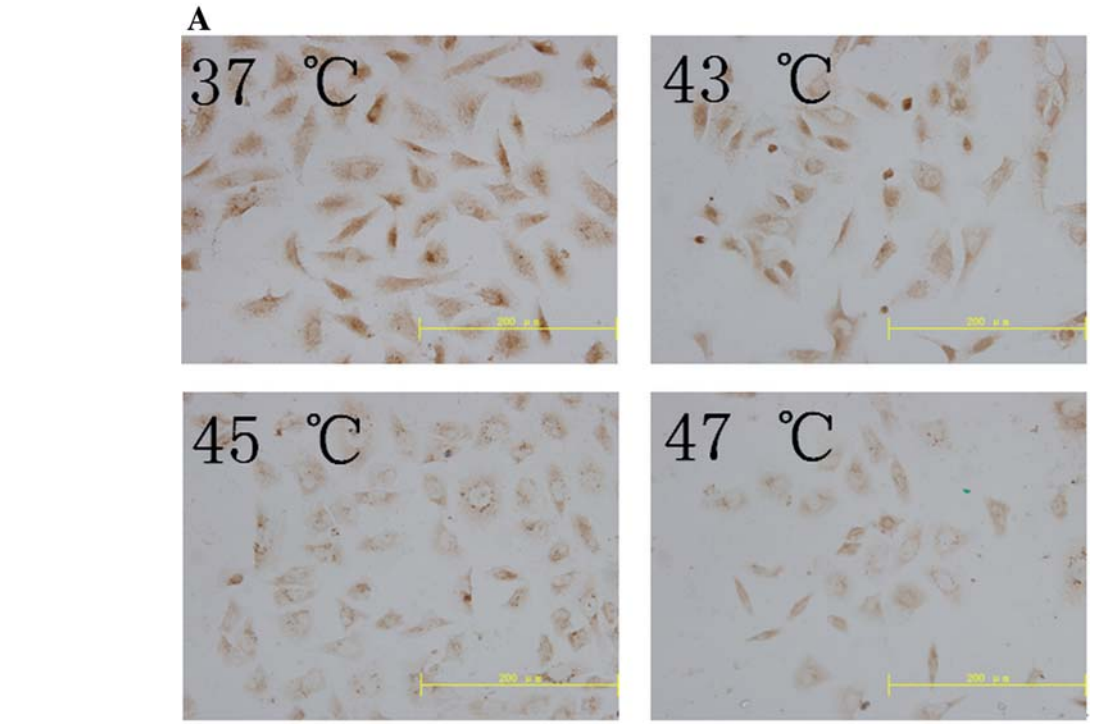


Figure 3A and B. Immunocytochemical imaging and corresponding column figure of TGF-$ß_1$ in MCF-7. (*) P<0.05 compared with control (n=5).

could be suppressed by hyperthermia. Figs. 3A and 4A show the photographs and corresponding average optical densities of TGF-$ß_1$ and VEGF after different temperature treatments. The immunocytochemical localization of TGF-$ß_1$ and VEGF in cytoplasm and cell membrane showed that the expression of TGF-$ß_1$ and VEGF in cells treated with hyperthermia for 30 min was significantly reduced compared with the control group (P<0.05). The reduction showed temperature dependence for VEGF, while differed insignificantly between each heating group for TGF-$ß_1$. Figs. 3B and 4B represent the corresponding average optical densities using Image-Pro Plus 6.0.

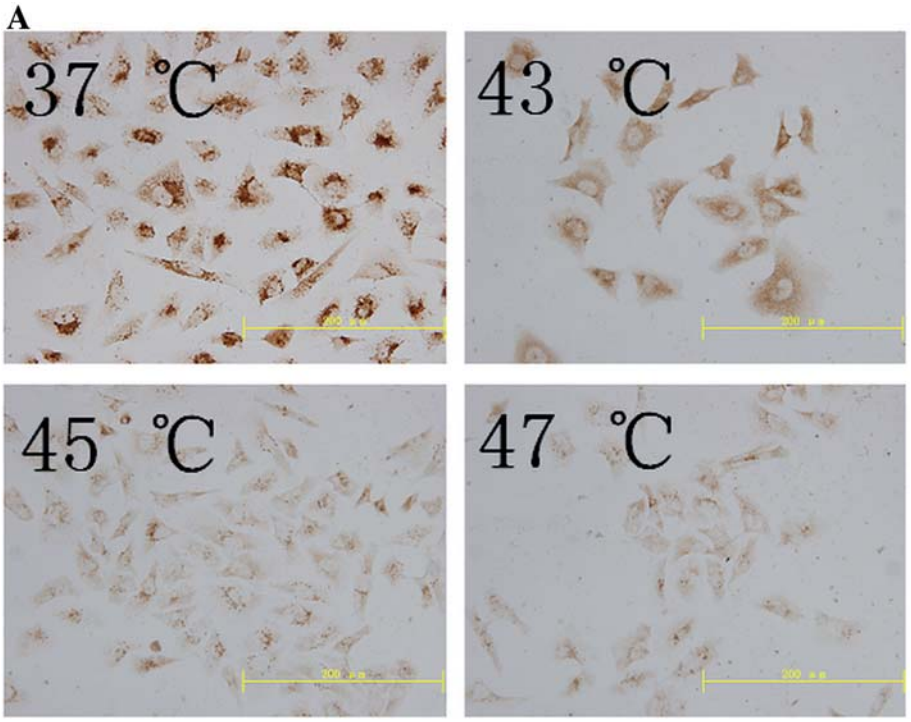
Down-regulation of TGF-$ß_1$ and VEGF mRNA expression. RT-PCR was performed to see whether the mRNA expression of TGF-$ß_1$ and VEGF in MCF-7 cells could be suppressed by hyperthermia. As shown in Figs. 5 and 6, the expression of TGF-$ß_1$ mRNA was significantly higher in control group (37˚C) than other heating groups. The expression of VEGF mRNA was significantly reduced by hyperthermia when temperature was above 43˚C.

Hyperthermia induces apoptosis. The Annexin/PI apoptosis detection kit was used to determine the percentage of apoptotic cells. Three populations of cells could be quantified: viable (no staining), early apoptotic (Annexin V^+, PI^-) and late apoptotic/necrotic (Annexin V^+ and PI^+). As shown in Fig. 7, proportions of early apoptotic cells in the 43 and 45 ˚C groups increased significantly and accounted for 6.82 and 8.45% (P<0.05). In the 45 and 47˚C groups, proportions of late apoptosis/necrosis cells significantly increased and accounted for 29.11 and 72.21%, respectively (P<0.05).

Discussion

Breast cancer is one of the leading causes of death in females. For women with local or regional disease, the 5-year survival rate may exceed 80%. Still, a significant number of patients relapse despite loco-regional treatments. Once distal metastasis has developed, the 5-year survival rate drops to only 26.7% (11). Metastasis is the final stage in tumor progression and is thought to be responsible for up to 90% of deaths associated with solid tumors (12). Although the local therapeutic effect of radiotherapy and surgery has been identified, the ability to suppress residual cells invasion and metastasis is insignificant. Even as an adjunctive therapy, other modalities that exert different anti-cancer mechanisms are desired. In the present study, we explored hyperthermia as a prospective therapy for breast cancer.

There is no doubt that as the temperature increases, the cytotoxicity of hyperthermia also increases. Our data of flow cytometric analysis show that higher temperature hyperthermia strengthened the lethal effect when compared to lower temperature. But with the rising therapeutic temperature, what would happen to the ability of invasion and metastasis?



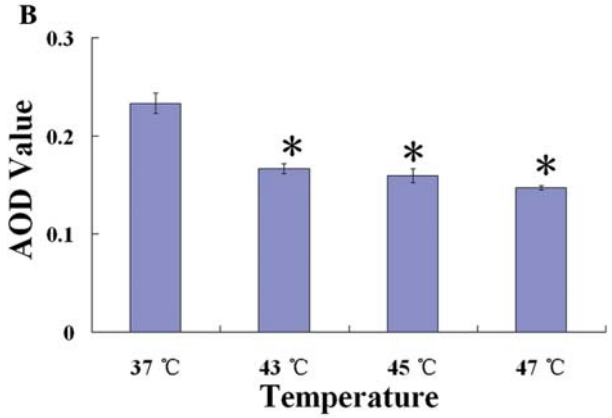


Figure 4A and B. Immunocytochemical imaging and corresponding column figure of VEGF in MCF-7. (*) P<0.05 compared with control; (Δ) P<0.05 compared with the 43˚C group (n=5).

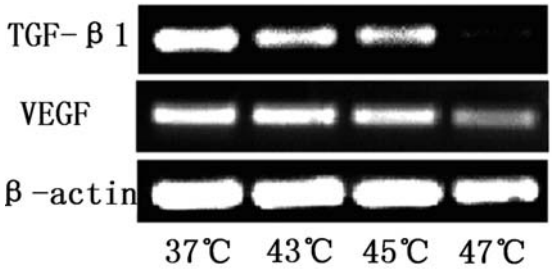


Figure 5. Expression of TGF-$ß_1$ and VEGF mRNA in MCF-7 cells.

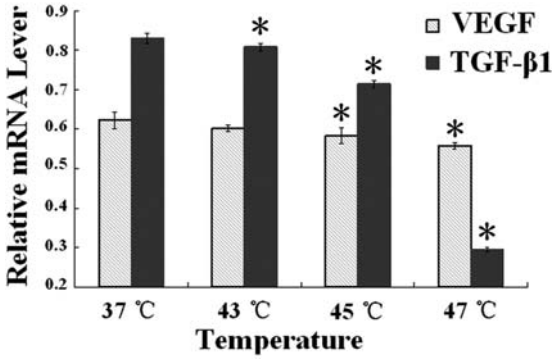


Figure 6. mRNA lever of TGF-$ß_1$ and VEGF. (*) P<0.05 compared with control; (n=3).

Which is the best temperature at which the tumor cells can be effectively killed while the invasion and metastasis ability suppressed? The present study is the first attempt to compare the effect on invasion ability of hyperthermia at different temperatures.

Our results indicated that hyperthermia at 43, 45 and 47˚C for 30 min could effectively inhibit tumor invasion *in vitro* which showed temperature dependence. This implies that hyperthermia with higher temperature may be more effective. It can be explained by not only cytotoxicity of hyperthermia but also some other mechanisms. To find out the possible underlying mechanism, we analyzed expression of TGF-$ß_1$, VEGF and MMP-2/9 in MCF-7 cells.

It is known that, degradation of basal lamina and extracellular matrix (ECM) is crucial for invasion and metastasis of malignant cells (13). Among the currently known 24 types of human MMPs (14), MMP-2 (or gelatinase A) is most frequently overexpressed in cancer and is instrumental in cutting through basement membrane barriers (15). It has been suggested that MMP-2 activation of HT-1080 cells is inhibited after heat shock at 42˚C for 3 h (12). Consistent with this finding, our study also presented similar effect in MCF-7 cells by heating at higher temperature and for shorter time.

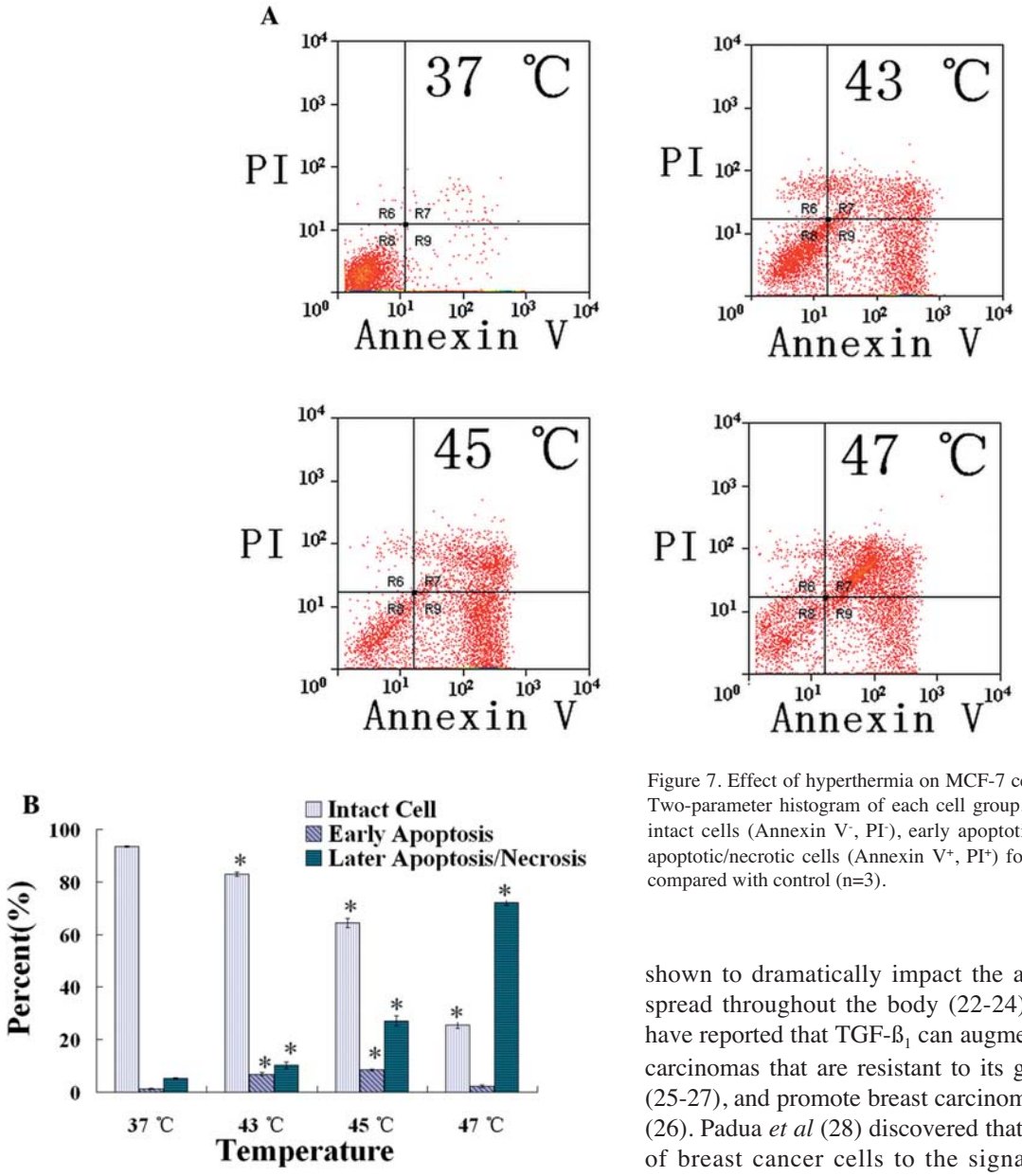


Figure 7. Effect of hyperthermia on MCF-7 cell necrosis and apoptosis. (A) Two-parameter histogram of each cell group. (B) Column data analysis of intact cells (Annexin V^-, PI^-), early apoptotic (Annexin V^+, PI^-) and late apoptotic/necrotic cells (Annexin V^+, PI^+) for each cell group. (*) $P<0.05$ compared with control (n=3).

Many studies have provided compelling evidence that VEGF plays an instrumental role in angiogenesis along with some of the pro-angiogenic factors that are known to have a contribution in the activation of MMP-2 (16). Upon ligand (VEGF) binding to its receptor VEGFR-2, tyrosine kinase signaling cascade is stimulated and migration factors such as MMPs are produced (17). In the present study, we demonstrated that hyperthermia treatment caused an inhibition in the protein and mRNA expression of VEGF in MCF-7 cells by heating at 43˚C or above 43˚C for 30 min. Other studies also suggested that hyperthermia reduced VEGF expression significantly by heating at 42˚C for 4 h (4). Here we deduce that down-regulation of VEGF expression in hyperthermia-treated MCF-7 cells may contribute to decrease MMP-2 activity in treated cells.

Several investigators demonstrated that TGF-$ß_1$ expression in a variety of malignancies was associated with increased tumorigenesis (18-21). The TGF-$ß_1$ pathway has been implicated in many of these metastatic processes, and it has been shown to dramatically impact the ability of tumor cells to spread throughout the body (22-24). Previous experiments have reported that TGF-$ß_1$ can augment the aggressiveness of carcinomas that are resistant to its growth inhibiting effects (25-27), and promote breast carcinoma metastasis to the bone (26). Padua *et al* (28) discovered that even transient exposure of breast cancer cells to the signaling molecule TGF-$ß_1$ promoted their extravasation from blood vessels and entry into the lung. These effects are closely associated with VEGF and/or MMP activities as mentioned above. Obviously, to understand the alteration of TGF-$ß_1$ in cancer with anti-cancer treatment is very important. However, there are few reports on TGF-$ß_1$ expression of carcinoma cells treated with hyperthermia. Our results firstly demonstrated that the hyperthermia at 43, 45 and 47˚C for 30 min down-regulated TGF-$ß_1$ mRNA and protein expression of MCF-7 cells. The results report here imply that expression of TGF-$ß_1$ in breast cancer could be used to judge the curative effect and prognosis for hyperthermia.

In our experiments, comparisons were made among 43, 45 and 47˚C and it was found that higher temperature hyperthermia strengthen the inhibiting effect of MMPs activity and VEGF expression when compared to lower temperature. However, there was no significant difference in TGF-$ß_1$ protein expression during 43, 45 and 47˚C. The discrepancy implies that the inhibition of MMPs may be multiply regulated by other factors. We suppose that down-regulation of TGF-$ß_1$, VEGF and MMP-2/9 of MCF-7 cells by hyperthermia treatment played an important role in the effect

ess invasion ability of residual cancer cells. , the associated mechanisms are so complicated that different anti-tumor effects may be observed when different temperature was delivered. Further studies are needed to elucidate the underlying mechanism.

In summary the data in the present study suggest that hyperthermia of higher temperature lasting for a certain period of time (47˚C for 30 min) achieved favorable anti-invasion effect, such effect may be due to its strong direct cytotoxicity and the down-regulation of TGF-$ß_1$, VEGF and MMP-2/9. Therefore, we believe that hyperthermia should also be regarded as an important therapy, which provides a new strategy for the treatment or combined treatment of breast cancer or even some other cancers which may metastasize at early stages and may be an important basis for further exploration in cancer therapeutics.

Acknowledgements

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