34th Annual International Conference of the IEEE EMBS
San Diego, California USA, 28 August - 1 September, 2012

A Novel Thermal Treatment Modality for Controlling Breast Tumor Growth and Progression

Yifan Xie, Ping Liu and Lisa X. Xu, *Senior Member, IEEE*

***Abstract*—The new concept of keeping primary tumor under control in situ to suppress distant foci sheds light on the novel treatment of metastatic tumor. Hyperthermia is considered as one of the means for controlling tumor growth. In this study, a novel thermal modality was built to introduce hyperthermia effect on tumor to suppress its growth and progression using 4T1 murine mammary carcinoma, a common animal model of metastatic breast cancer. A mildly raised temperature (i.e.39℃) was imposed on the skin surface of the implanted tumor using a thermal heating pad. Periodic heating (12 hours per day) was carried out for 3 days, 7 days, 14 days, and 21 days, respectively. The tumor growth rate was found significantly decreased in comparison to the control without hyperthermia. Biological evidences associated with tumor angiogenesis and metastasis were examined using histological analyses. Accordingly, the effect of mild hyperthermia on immune cell infiltration into tumors was also investigated. It was demonstrated that a delayed tumor growth and malignancy progression was achieved by mediating tumor cell apoptosis, vascular injury, degrading metastasis potential and as well as inhibiting the immunosuppressive cell myeloid derived suppressor cells (MDSCs) recruitment. Further mechanistic studies will be performed to explore the quantitative relationship between tumor progression and thermal dose in the near future.**

I. Introduction

Breast cancer was known to affect more than 200,000 women in the United States alone in 2010[1]. It is the leading cause of cancer death in women.

Treatments include surgery, radiotherapy, chemotherapy, gene therapy etc. But recurrent rate of malignant tumor is still high[2], and the efficacy of the existing therapeutic means is yet to be improved. A new concept has been proposed recently that primary tumor has suppressive effect on distant foci [3, 4]. This sheds light on tumor treatment. Keeping the primary tumor in situ but restricting its size may enable the host to impede the development of distant foci. Thermal therapy has been attempted to locally destroy tumor cells and enhance the body defense against tumor cells.

*Research supported by The National Basic Research Program of China (973 Program 2010CB834303) and Shanghai Science and Technology Committee (11DZ2211000).

Y. Xie (e-mail: xieyifan816@yahoo.com.cn) is with the School of Biomedical Engineering, Shanghai Jiao Tong University, Shanghai, People's Republic of China.

P. Liu (co-corresponding author; e-mail: pingliu@sjtu.edu.cn) is with the Med-X Research Institute, Shanghai Jiao Tong University, Shanghai, People's Republic of China.

L. Xu (co-corresponding author; e-mail: lisaxu@sjtu.edu.cn) is with the Med-X Research Institute and School of Biomedical Engineering, Shanghai Jiao Tong University, Shanghai, People's Republic of China.

Thermal treatment utilizes energy intervention through freezing or heating the malignant tissues for tumor therapy. Intervening the tumor growth and progression via thermal energy infusion is worthy attempting. Hyperthermia, mildly heating cells up to about 40-45℃ changes cell membrane fluidity, cytoskeletal protein structures, and impedes trans-membrane protein functions. A series of physical and biochemical changes caused inside the cells during hyperthermia may lead to cell necrosis or apoptosis[5]. As a promising cancer therapy other than chemotherapy and radiotherapy, hyperthermia becomes more important than ever. Early studies focused upon the cytotoxic effects of high temperatures and the direct killing of tumor cells (＞42℃), but the application, measurement and consistency of the temperature range within cancer clinical trials had encountered some problems, i.e. accurate thermal dose for effective tumor therapy [6]. Accordingly, effects of mild temperature hyperthermia (defined as 39-41℃, within the fever range) on tissues were ignored. Although the imposed thermal dose through this mild hyperthermia was not enough to directly kill most of tumor cells, mild temperature is readily achievable and tolerated, and several randomized clinical trials demonstrated that mild effectively enhanced the response of tumor to radiotherapy, and improved local tumor control and patient survival[7-10], and both preclinical and clinical data results have demonstrated improved antitumor immune responses within the mild hyperthermia[11]. Many researchers designed experiments to determine thermal dose in hyperthermia[12], but few focused on the relationship between tumor and energy, especially for a long-term local hyperthermia treatment. One clinical trial indicated the usefulness of long-term hyperthermia therapy for maintaining performance status, symptomatic improvement, and prolongation of survival time in patients with peritoneal dissemination[13]. In another clinical case, it was reported that treatment including radiotherapy and neoadjuvant chemotherapy plus local hyperthermia was performed over 12 months. The result showed long-term hyperthermia might be useful in the treatment of local breast cancer[14].

Based on these previous clinical studies, we proposed that long-term hyperthermia was used as an energy infusion to perturb tumor growth and progression. In this study, tumor growth and progression were studied using 4T1 murine mammary carcinoma, a common animal model of metastatic breast cancer. The temperature 39°C was chosen in a range which was endurable for mice and could potentially affect tumor growth as well. A heating device was designed and attached to the mouse to achieve local hyperthermia on tumor every 12 hours during night time without affecting the normal mouse activities. After different periods of heating,

histological examination was performed to observe the therapeutic effect, mild hyperthermia mediating tumor cell apoptosis, tumor vasculature, metastasis potential and the anti-tumor immune response were also evaluated.

II. MATERIALS & METHODS

A. Animal models

6-8 week old female BALB/c mice were purchased from the Animal Laboratory of Shanghai Medical College, China. They were housed in isolated cages with a 12h light/dark cycle environment and fed with sterile food. The murine breast carcinoma cell line 4T1 was obtained from Shanghai First People's Hospital, China. Cells were maintained at 37℃ in a mixture of 5% CO_2 and 95% air in RPMI 1640 medium (Hyclone, USA) supplemented with 10% fetal bovine serum. To prepare the tumor-bearing mouse, mice were anesthetized using sodium pentobarbital intraperitoneally, approximately 1×10^6 cells were injected subcutaneously into the back region of each mouse. Mice were randomly assigned into two groups: tumor bearing control and mild hyperthermia treatment. Local hyperthermia treatment was started on the 7th day after tumor implantation. Tumors were then measured with a vernier caliper to determine the length, width and height, the tumor volume was calculated by using the formula:

$$V(mm^3) = \frac{\pi}{6} \times length \times width \times height$$

The volumes of tumors in both treated mice and control mice were measured on daily basis.

B. Long-term mild hyperthermia treatment

The body temperature of mouse is normally about 35-36℃, which is much lower than other animals. A mild temperature of 39℃ was imposed on the skin surface of the implanted tumor. Periodic heating (12 hours per day) was carried out for 3 days, 7 days, 14 days or 21 days, respectively. Tumors were removed from the mice immediately after the treatment. Tumor tissues were snap frozen in cold isopentane and stored at -80℃.

The hyperthermia system consisted of: resistor heating chip, temperature collection and control part.

1) Heating part

Electro resistor heating chips were designed with an outer diameter of 15 mm. The effective heating area was around 10 mm, with the average heating power of 0.5 W/cm^2. The heating chip was then wrapped in a cloth-make pocket and tied around the mouse body against to the tumor. An adjustable DC power supply was used to elevate the temperature.

2) Data monitoring, collection and control

One T-type thermocouple was inserted between the heating chip and the tumor to detect temperature. The signals from the thermocouple were collected and input to Agilent 34970A. The control modality was composed by the 34907A module of Agilent and an electromagnetic relay, performing on-off actions according to the temperature control algorithms. Labview was used to control the whole system.

C. Preparation of tumor tissue samples

The frozen tumor tissues were cut along the sagittal direction of skin using a LEICA CM1900 at -20℃, 20μm in thickness, then was placed on a microscope glass. Slides were stored frozen until use.

D. Detection of apoptosis

Apoptosis was detected using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore). Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) is a method of choice for rapid identification and quantification of the apoptotic cell fraction in tissues. Cryo-sections were fixed in 4% paraformaldehyde. TdT enzyme was then added for 1 hour at 37℃ to label fragmented DNA ends with digoxigenin nucleotides. Anti-digoxigenin antibody that conjugated to a peroxidase reporter molecule was then added. Methyl green was for counterstain. The slides were observed using light microscopy.

E. Immunohistochemistry

For immunohistochemistry, tumor slices from mouse biopsies were allowed to dry for 1 hour at room temperature, and fixed for 10 minutes in cold acetone, then permeabilized with 0.1% Triton X-100, slides were peroxidase quenched in 0.3% H_2O_2 in methanol, washed 3 times for 2 minutes in PBS, and then blocked for 30 minutes in 5% Bovine Serum Albumin (BSA). Primary antibodies, anti-MMP9 (Abcam, 1:1000), were applied overnight at 4℃ in PBS, followed by washes in PBS, and then biotinylated secondary antibody, streptavidin-HRP and diaminobenzidine (DAB) peroxidase substrate were added. Nuclei were counterstained with hematoxylin.

F. Immunofluorescence staining

For immunofluorescence staining, slides were first air dried for 1 hour at room temperature, and fixed 10 minutes in cold acetone, washed 3 times for 2 minutes in PBS, and then blocked for 1 hour in 10% Bovine Serum Albumin (BSA). Primary antibodies, FITC-conjugated anti-mouse CD11b antibody (BioLegend, 1:200) and PE-conjugated anti-mouse Gr-1 antibody (BioLegend, 1:400) or anti-mouse CD31 (R&D, 1:200), were applied overnight at 4℃. VECTASHIELD Mounting Medium with DAPI (Vector Laboratories) was used as a counterstain. Slides were viewed using a confocal microscope (Leica TCS SP5).

III. RESULTS

A. Long-term mild hyperthermia treatment delays tumor growth in 4T1 breast tumor

Mild hyperthermia was applied to tumors on day 7 after tumor cell injection and carried out for 3 days, 7 days, 14 days and 21days, respectively. The corresponding mean tumor volumes measured are given in Fig. 1. As illustrated, tumors in control group grew significantly faster than the treated tumors, which indicated that the inhibition effect of the elevated temperature on the 4T1 tumor growth.

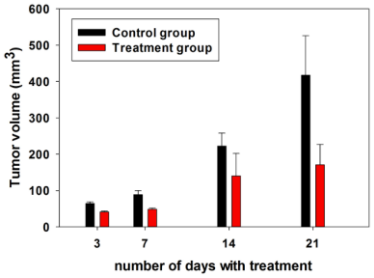

Figure 1. Treatment with mild hyperthermia was initiated in 4T1 murine breast carcinoma on day 7 post tumor cell injection and carried out for 3 days, 7 days, 14days and 21days. Mean tumor volume after 3, 7, 14, 21 days (n=3/group) was displayed.

B. Mild hyperthermia mediated apoptosis

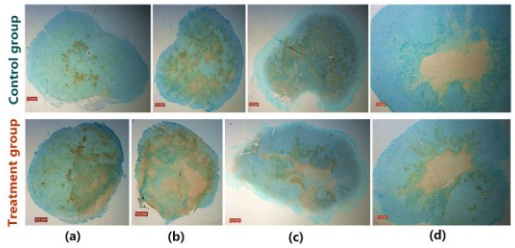

Figure 2. Mild hyperthermia mediated apoptosis. Apoptotic cells were visualized in frozen sections from 3 days (a), 7 days (b), 14 days (c) and 21 days (d) groups using the TUNEL method. Apoptotic cell manifests a brown TUNEL-positive nuclear signal. Slides were observed on a stereoscope. Scale bar: 0.5mm.

Cryosections of tumor tissues taken in 3 days (Fig. 2a), 7 days (Fig. 2b), 14 days (Fig. 2c) and 21 days (Fig. 2d) of the treatment were used for apoptosis analyses. Numbers of apoptotic cells were significantly increased in the treated tumor (3days and 7days) than that of the control. The results suggested that mild hyperthermia mediated apoptosis that is associated with the initial suppression of tumor growth in the treated groups as shown in Fig.1.

C. Long-term mild hyperthermia reduces vascular area in the 4T1 breast tumor

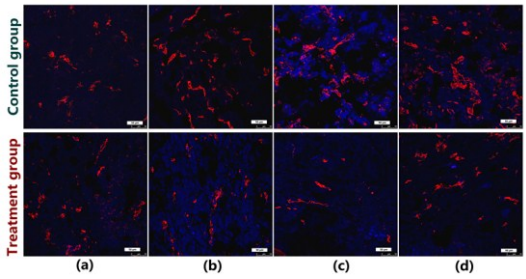

Figure 3. Tumor sections from 3 days (a), 7 days (b), 14 days (c) and 21 days (d) groups were analyzed by immunofluorescence using CD31, an endothelial cell marker, red; Nucleolus, blue. Total magnification, 400×. Scale bar: 50μm.

To determine the effect of long-term mild hyperthermia on angiogenesis, vascular area (positive fluorescent area/400× field) was assessed on the treatment groups of different lengths. Shown in Fig. 3, on the 3rd day, no obvious difference was found between treatment group and control group. But, on other time points, control tumor tissues increased in vascular area, comparing to the treated tumor tissues (Fig.4). The most significant difference was observed on the 21st day of the treatment. In conclusion, long-term mild hyperthermia was effective in decreasing vascular density in 4T1 breast tumor.

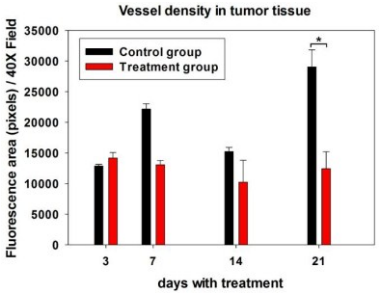

Figure 4. Vascular area was evaluated. Data are displayed as mean±SEM and represents 5 images (total magnification, 400×) per tumor and three tumors per group. *P<0.05.

D. Effect of long-term mild hyperthermia on MDSC infiltration in the 4T1 breast tumor

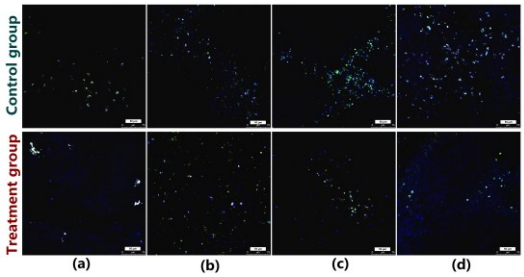

Figure 5. Infiltration of MDSCs in the 4T1 tumor sections by immunofluorescence. MDSCs defined as co-localization of CD11b+ and Gr-1+ cells. (a) 3 days, (b) 7 days, (c) 14 days and (d) 21 days group. CD11b+, red; Gr-1+, green; Nucleolus, blue. Total magnification, 400×. Scale bar: 50μm.

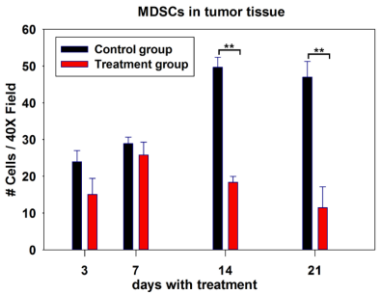

Figure 6. Tumor sections were evaluated MDSCs defined as the number of cells that express CD11b and Gr1 per 400× field. Data are displayed as mean±SEM and represents 5 images (total magnification, 400×) per tumor and three tumors per group. **P<0.01.

The effect of mild hyperthermia on immunosuppressive cells MDSCs (CD11b+Gr1+ cells) infiltration into tumors was investigated. There was a significant decrease in MDSC infiltration of treated tumors compared to control tumors (Fig. 5). Tumor sections were evaluated for MDSCs defined as the number of cells which express CD11b and Gr1 per 400× field (Fig. 6), MDSCs increased in control tumor, however the treated tumor had a significant reduction in MDSC infiltration

compared to control, indicating that long-term mild hyperthermia was involved in inhibiting immunosuppressive cells recruitment.

E. Immunohistochemical staining MMP-9 expression in 4T1 tumor tissue at different treatment stages

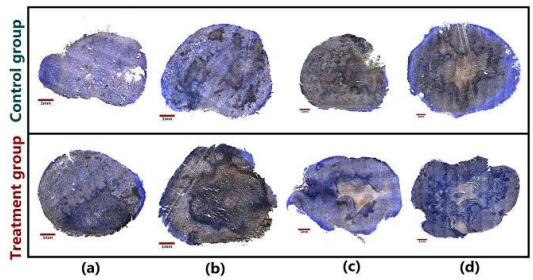

Figure 7. Immunohistochemical analysis of MMP9 expression in 4T1 tumor tissue at different treatment stages. Tumor sections were used from 3 days (a), 7 days (b), 14 days (c) and 21 days (d) treatment. MMP9-positive, dark-brown; Nucleolus, blue. Slides were photographed on optical microscope (total magnification, 40X) and spliced. Scale bar: 1mm.

MMP9 expression was analyzed by immunohistochemical in 4T1 tumor tissue at different treatment stages. There was a significantly increased MMP9 expression during the tumor growth in control tumor, however. Increased MMP9 expression was found in the treated tumor on the 3rd, 7th day, but on the 14th day, there were significant reduction in MMP9 expression (Fig. 7), indicating mechanistic change in tumor metastasis potential induced by hyperthermia, which is worthy to be further investigated.

IV. Discussion and Conclusion

In this study, a long-term hyperthermia at 39°C was imposed on the mice tumor to investigate the effect of the thermal energy on tumor growth and progression. Results indicated that long-term hyperthermia could have an accumulative effect on tumor cell proliferation via sustained thermal energy supply, delayed tumor growth and malignancy progression was found. This might be attributed to sustained thermally induced cell apoptosis, and reduction of tumor vasculature. Some research supported that mild hyperthermia could improve antitumor immune responses [15, 16]. MDSCs promote tumor progression through inhibiting both innate and adaptive antitumor immunity by a variety of pathways[17]. Histological analyses indicated that a significant decrease in MDSC infiltration of treated tumors as compared to tumors in control, which suggested the underlying mechanism associated to the hyperthermia enhanced antitumor immune response. Also, MMP9 plays a key role in tumor progression and metastasis, specifically in the degradation of the extracellular matrix and assists cancer cell invasion[18].Our results showed that decreased expression of MMP9 on the 14th and 21st day of treatment, suggesting that sustained mild hyperthermia might reduce the metastasis potential of tumor cell. In light of this, a novel thermal treatment modality could be developed using fever-range hyperthermia for in situ tumor suppression and combined with other anti-cancer therapies, including immunotherapy and chemotherapy in future study.

Reference

[1] A. Jemal, R. Siegel, J. Xu, and E. Ward, "Cancer statistics, 2010," *CA Cancer J Clin,* vol. 60, pp. 277-300, Sep-Oct 2010.

[2] M. Clarke, R. Collins, S. Darby, C. Davies, P. Elphinstone, E. Evans, J. Godwin, R. Gray, C. Hicks, S. James, E. MacKinnon, P. McGale, T. McHugh, R. Peto, C. Taylor, and Y. Wang, "Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials," *Lancet,* vol. 366, pp. 2087-106, Dec 17 2005.

[3] B. Fisher, N. Gunduz, and E. A. Saffer, "Influence of the Interval between Primary Tumor Removal and Chemotherapy on Kinetics and Growth of Metastases," *Cancer Research,* vol. 43, pp. 1488-1492, April 1, 1983.

[4] K. Camphausen, M. A. Moses, W. D. Beecken, M. K. Khan, J. Folkman, and M. S. O'Reilly, "Radiation therapy to a primary tumor accelerates metastatic growth in mice," *Cancer Res,* vol. 61, pp. 2207-11, Mar 1 2001.

[5] L. X. Xu, A. Zhang, P. Liu, C. Chen, J. Sun, and D. M. Sabados, "Energy-based diagnostic and treatment techniques," *IEEE Eng Med Biol Mag,* vol. 27, pp. 72-7, Sep-Oct 2008.

[6] M. W. Dewhirst, Z. Vujaskovic, E. Jones, and D. Thrall, "Re-setting the biologic rationale for thermal therapy," *Int J Hyperthermia,* vol. 21, pp. 779-90, Dec 2005.

[7] C. C. Vernon, J. W. Hand, S. B. Field, D. Machin, J. B. Whaley, J. van der Zee, W. L. van Putten, G. C. van Rhoon, J. D. van Dijk, D. Gonzalez Gonzalez, F. F. Liu, P. Goodman, and M. Sherar, "Radiotherapy with or without hyperthermia in the treatment of superficial localized breast cancer: results from five randomized controlled trials. International Collaborative Hyperthermia Group," *Int J Radiat Oncol Biol Phys,* vol. 35, pp. 731-44, Jul 1 1996.

[8] J. van der Zee, D. Gonzalez Gonzalez, G. C. van Rhoon, J. D. van Dijk, W. L. van Putten, and A. A. Hart, "Comparison of radiotherapy alone with radiotherapy plus hyperthermia in locally advanced pelvic tumours: a prospective, randomised, multicentre trial. Dutch Deep Hyperthermia Group," *Lancet,* vol. 355, pp. 1119-25, Apr 1 2000.

[9] Y. Harima, K. Nagata, K. Harima, V. V. Ostapenko, Y. Tanaka, and S. Sawada, "A randomized clinical trial of radiation therapy versus thermoradiotherapy in stage IIIB cervical carcinoma," *Int J Hyperthermia,* vol. 17, pp. 97-105, Mar-Apr 2001.

[10] W. G. Kraybill, T. Olenki, S. S. Evans, J. R. Ostberg, K. A. O'Leary, J. F. Gibbs, and E. A. Repasky, "A phase I study of fever-range whole body hyperthermia (FR-WBH) in patients with advanced solid tumours: correlation with mouse models," *Int J Hyperthermia,* vol. 18, pp. 253-66, May-Jun 2002.

[11] J. J. Skitzki, E. A. Repasky, and S. S. Evans, "Hyperthermia as an immunotherapy strategy for cancer," *Curr Opin Investig Drugs,* vol. 10, pp. 550-8, Jun 2009.

[12] S. A. Sapareto and W. C. Dewey, "Thermal dose determination in cancer therapy," *Int J Radiat Oncol Biol Phys,* vol. 10, pp. 787-800, Jun 1984.

[13] H. Minakuchi, R. Hirayama, S. Sawai, Y. Kawachi, S. Tominaga, Z. Nihei, and Y. Mishima, "Clinical trials of long-term RF local hyperthermia for advanced gastric cancer," *Jpn J Surg,* vol. 20, pp. 238-9, Mar 1990.

[14] V. V. Ostapenko, M. Yamazaki, T. Nishide, H. Tanaka, M. Miyano, M. Sonobe, K. Toda, M. Mune, I. Nishide, and S. Yukawa, "Long-term local hyperthermia in the treatment of advanced breast cancer (case report)," *Anticancer Res,* vol. 21, pp. 4117-9, Nov-Dec 2001.

[15] S. K. Calderwood and D. R. Ciocca, "Heat shock proteins: Stress proteins with Janus-like properties in cancer," *International Journal of Hyperthermia,* vol. 24, pp. 31-39, 2008.

[16] C. J. Turtle and D. N. Hart, "Dendritic cells in tumor immunology and immunotherapy," *Curr Drug Targets,* vol. 5, pp. 17-39, Jan 2004.

[17] S. Ostrand-Rosenberg and P. Sinha, "Myeloid-derived suppressor cells: linking inflammation and cancer," *J Immunol,* vol. 182, pp. 4499-506, Apr 15 2009.

[18] M. Egeblad and Z. Werb, "New functions for the matrix metalloproteinases in cancer progression," *Nat Rev Cancer,* vol. 2, pp. 161-74, Mar 2002.