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Study of Thermal Effect on Breast Tumor Metabolism and Growth Using Metabonomics

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Abstract— **In this study, the biological effects of long-term mild hyperthermia treatment on tumor metabolism and growth were investigated using 4T1 murine mammary carcinoma, a common animal model of metastatic breast cancer. Periodic thermal treatment (12 hours per day) was applied to tumors and carried out for 3 days, 7 days, 14 days, and 21 days, respectively. The metabolites of tumor tissues were analyzed by gas chromatography-mass spectrometry. The results showed that the growth rate of thermally treated tumors was inversely related to the abundance of long chain fatty acids and acyl glycerols identified in tumor tissues. In the first two weeks, the growth of thermally treated tumors was significantly inhibited, while there was an obvious accumulation of long chain fatty acids and acyl glycerols in tumor tissues. In the third week, the thermally treated tumors adapted to the thermal environment and started to regrow, while the abundance of long chain fatty acids and acyl glycerols decreased in the tumor tissues. These observations suggested that the blockade of long chain fatty acid synthesis during mild hyperthermia treatment of tumors could improve the long-term treatment effect by limiting the supply of substance and energy for tumor re-growth.**

I. Introduction

According to American Cancer Society, approximately 230,480 new cases of invasive breast cancer were estimated among US women in 2011, making it the most dangerous malignant cancer in women [1]. Global researchers are exploring the effective carcinoma treatment and hyperthermia has become one in tumor physical therapy. Previous researches indicated that thermal treatment at high temperatures could kill tumor cells by necrosis, while heating at relatively mild temperatures could kill tumor cells by apoptosis [2]. Besides, randomized clinical trials showed that hyperthermia could also enhance the effect of chemotherapy and radiotherapy [3, 4], and improve antitumor immune responses [5].

Over the past decades, researchers have been studying the biological mechanism of tumor thermal treatment from the perspectives of protein and gene expressions [6]. Metabonomics, a new methodology arising from the post-genomics era, is a high-throughput and global approach in addition to proteomics and genomics for analysis of molecular changes [7]. It provides perspectives into the drug, disease or environment change by identifying and modeling the changes of metabolites found in biological fluids, tissues or urine samples with NMR, GC-MS and LC-MS as the main research methodologies [8-9]. Many researchers had applied these technologies to study the biological mechanisms of cancer. Denkert et al. performed GC-TOF MS profiling to analyze the metabolism between invasive ovarian carcinomas and ovarian borderline tumors. They found that creatinine, lactate and tricarboxylic acid cycle intermediates were elevated in ovarian cancer which indicated that malignant tumors had higher metabolic turnover rates and higher demands in energy supply [10].

The present work studied the effect of the long-term mild hyperthermia treatment on the growth and metabolism of breast tumor. BALB/C 4T1 tumor-bearing mice were used for breast carcinoma model, and the self-designed heating pads were placed on tumor surface to impose a local thermal environment around the tumor at a constant temperature for 12 hours every day. After different periods of thermal treatment, the tumor tissues were resected for the preparation of metabonomics analysis. According to the animal experiment results, thermal treatment delayed tumor growth in the first two weeks, but the thermally treated tumors recovered the vigorous growth in the third week. GC-MS technology coupled with the statistical analysis was used to investigate the metabolic alteration of tumor tissues. Interestingly we found that the abundance of long chain fatty acids and acyl glycerols in the thermally treated tumor tissues accumulated in the first two weeks and declined in the third week. Based on these results, we proposed that the accumulation of long chain fatty acids and acyl glycerols in the thermal treatment group played an important role in supporting tumor re-growth.

II. Materials & Methods

A. Animal models

6-8 weeks old female BALB/c mice (20 ± 2 g) were bought from the Animal Laboratory of Shanghai Medical College, China. They were housed in sterile isolated cages with a 12 h light/dark cycle environment and fed with sterile food and water. The murine breast carcinoma cell line 4T1 was obtained from Shanghai First People's Hospital, China. Cells were maintained at 37 °C in a mixture of 5% CO_2 and 95% air in RPMI 1640 medium supplemented with 10% fetal bovine serum. To prepare the tumor-bearing mice, they were

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anesthetized using sodium pentobarbital intraperitoneally, and 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[11].

B. Long-term mild hyperthermia treatment

The hyperthermia system consisted of: resistor heating pad, temperature collection and control part. Electro resistor heating pads 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 pad 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. One T-type thermocouple was inserted between the heating pad 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. As one knows that the body temperature of mice is commonly around 35-36 °C. Considering that the range of hyperthermia is less than 5 °C above the body temperature, we selected the treatment temperature at 39 °C as the heating temperature, imposed on the skin surface of the implanted tumor, which was bearable of mice. 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 frozen in cold isopentane and stored at -80 °C [11].

C. Preparation of tumor tissue samples

Each tissue sample (50 mg) was pulverized after being frozen in liquid nitrogen with the addition of 250 μL of mixed solvent (chloroform/methanol/water = 1:2.5:1, v/v/v).The lysate was ultrasonicated for 1 min and stored at -20 °C for 20 min, then centrifuged at 15700 g (4 °C) for 10 min. A total of 150 μL of aqueous supernatant was transferred to a GC vial containing two internal standards, L-2-chlorophenylalanine (10 μL, 0.3 mg/mL) and heptadecanoic acids (10 μL, 1.0 mg/mL). The deposit was rehomogenized with a T10 basic homogenizer (IKA, Staufen, Germany) for 30 s at 0 °C after adding 250 μL of methanol. After a second centrifugation, another 150 μL aliquot of supernatant was added to the mixture in the GC vial and vacuum-dried. The residue was derivatized using a two-step procedure. First, 80 μL of methoxyamine (15 mg/mL in pyridine) was added to the vial and kept at 30 °C for 90 min, followed by 80 μL of BSTFA (1% TMCS) at 70 °C for 60 min.

D. GC-MS analysis

The derivatized tissue samples were analyzed by an Agilent 7890A gas chromatography system coupled to an Agilent 5975C MSD system with Triple-Axis Detector (Agilent, CA) as described in previous published literature except that the solvent delay time was set to 6.5 min. A HP-5MS fused-silica capillary column (30 m × 250 μm i.d.; Agilent J & W Scientific, Folsom, CA) was utilized to separate the trimethylsilylated derivatives. Helium was used as a carrier gas with a constant flow rate of 1 mL/min. One microliter of derivatives was injected, and the solvent delay time was set to 6.5 min. The initial oven temperature was held at 60 °C for 2 min, ramped to 140 °C at a rate of 10 °C/min, to 240 °C at a rate of 4 °C/min, to 300 °C at a rate of 10 °C/min, and finally held at 300 °C for 8 min. The initial inlet gas pressure was 8.23 psi. The temperatures of injector, transfer line, and electron impact (EI) ion source were set to 250 °C, 290 °C, and 230 °C, respectively. The electron energy was 70 eV, and mass data was collected in a full scan mode (m/z 50-600). Agilent "retention time locking" (RTL) was applied to control the reproducibility of retention times (RT).

E. Data extraction and preprocessing

Raw GC-MS data files were exported to netCDF files in Agilent MSD ChemStation software. The netCDF files were introduced to the TagFinder software for baseline filtering and peak detection one by one, and the resulting data of each sample was exported to a single text file. Then the separate text files were imported to TagFinder software, where retention time correction, peak alignment, mass tag correlation, clustering, and grouping were performed. The tag output was further processed in Microsoft Excel 2007. Referring to the data of the blank derivatization sample, all artificial noise signals produced during the processes of solvent extraction, derivatization, and GC-MS analysis were excluded. As a result, only the information from base peak ion of each cluster (one compound) was kept as quantifier. The resulting data consisting of arbitrary peak indexes (RT-m/z pair), sample names (observations), and peak areas (variables) were exported for multivariate statistical analysis.

F. Identification of metabolites

The deconvoluted spectra were introduced to the NIST MS Search 2.0 software for automatically searching compound information from the NIST 08 library and the author-constructed standard library. The searching results with match similarity larger than 80% will be accepted as candidate compounds. To obtain reliable results, the representative GC/MS data files of two groups were imported to the AMDIS software for automatically searching against an author-constructed standard library including retention time and mass spectra. The results obtained from the above two methods were confirmed as reliable candidate compounds. The retention times of reference standards were utilized to confirm the candidate compounds. The Kovats retention indexes of remaining candidate compounds were calculated and compared with a NIST RI database and available internet database such as NIST Chemistry WebBook.

G. Statistical analysis

The resulting data was analyzed in the SIMCA-P 12.0 Software package (Umetrics, Umea, Sweden) and SPSS 17.0 (IBM, USA). Comparisons of data between two groups were analyzed by Student's t test and were considered significant when two-tailed $p < 0.05$.

III. Results and Discussion

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

Mild hyperthermia was applied to tumors on the 7th day after 4T1 cell injection and carried out for 3 days, 7 days, 14 days and 21 days, respectively. The average tumor mass was given in Fig. 1. As illustrated, the average mass of tumors at the same time points in the thermal treatment group was lighter than that in the control group, especially on the 14th day. However, the thermally treated tumors recovered the vigorous growth in the third week. It suggested that the thermal treatment could inhibit tumor growth in the first two weeks, but could not inhibit its growth persistently.

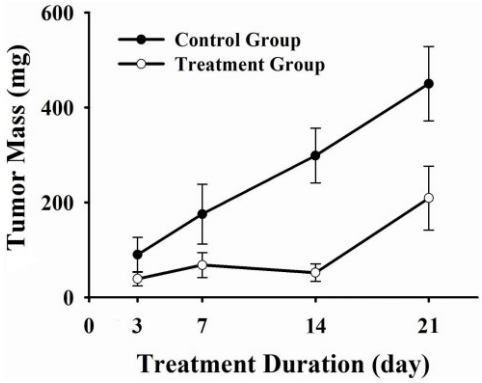

Figure 1. The average tumor mass of control group and thermal treatment group.

B. Overview and classification of metabolites identified

80 peaks were identified as the endogenous metabolites, and were used to build the 80-dimensional vector to characterize the metabolic profile of tumor tissues. We classified the 80 identified metabolites into six groups by the pathways, namely long chain fatty acids, acyl glycerols, organic acids, amino acids, monosaccharides and others. Besides, we counted the fold change of peak intensities of the metabolites between the thermal treatment group and control group at the same time points (Fig. 2). It showed that the abundance of long chain fatty acids and acyl glycerols were upregulated in the thermal treatment group versus that of the control group on the 14th day.

C. Highlighted metabolites by statistical analysis

In this part, partial least square discrimination analysis (PLS-DA) was performed and a measurement named VIP (variable importance for the projection) which summarized the importance of the variables (metabolites) had been calculated. In general, VIP values larger than 1 indicated important variables. We further identified the metabolites which were significantly upregulated in the thermal treatment group versus that of the control group on the 14th day according to the following criteria: (1) fold change > 1.5; (2) p-value < 0.05; (3) VIP > 1. The results showed that the long chain fatty acids and acyl glycerols had the most significant differences between the thermal treatment group and control group on the 14th day (Table. 1).

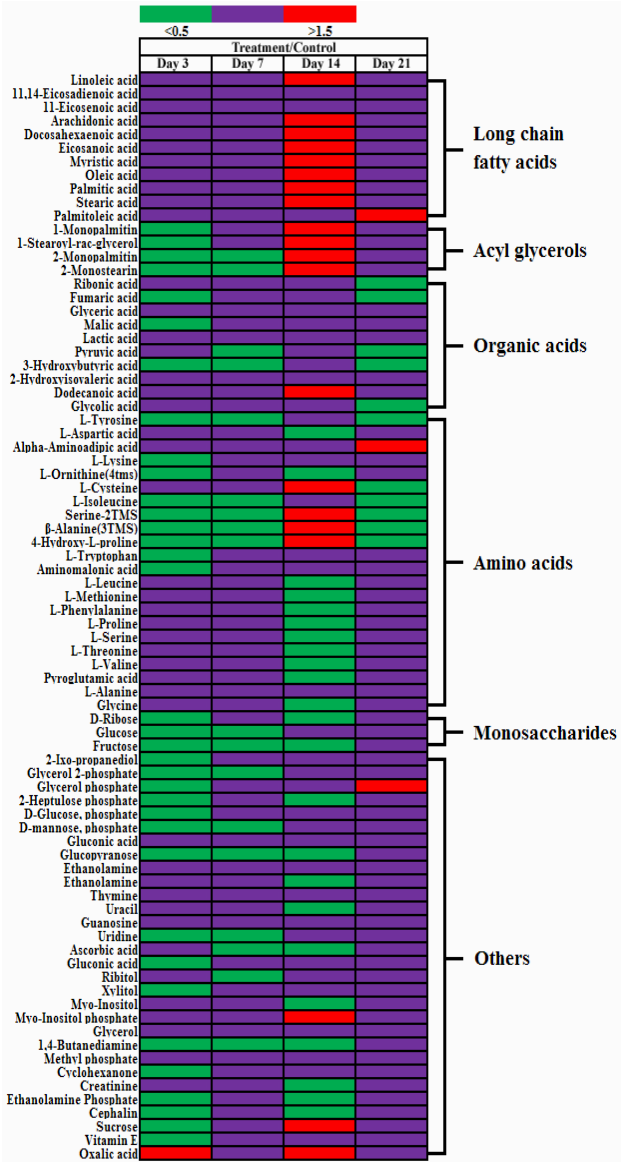

Figure 2. Overview and classification of metabolites identified.

D. The correlation between tumor mass and total abundance of long chain fatty acids and acyl glycerols

Compared to the control group, both the tumor growth and metabolism showed significant differences in the thermal treatment group on the 14th day. We summed the abundance of all identified long chain fatty acids and acyl glycerols, and compared them with the tumor mass at the four time points (Fig. 3). It indicated that the tumor mass was inversely related to the total abundance of long chain fatty acids and acyl glycerols identified in tumor tissues. In the control group, as the rapid growth of tumor, the abundance of long chain fatty acids and acyl glycerols identified in tumor tissues showed a declining trend. In the thermal treatment group, there was a remarkable accumulation of long chain fatty acids and acyl glycerols in the tumor tissues due to the slow tumor growth in the first two weeks, while the abundance of long chain fatty acids and acyl glycerols in the tumor tissues declined in the third week along with the re-growth of tumors.

Table 1. THE UPREGULATED MEATBOLITES IN THE THERMAL TRETMENT GROUP ON THE 14TH DAY

Metabolites	FC	P-value	VIP
Linoleic acid	2.92	6.97E-03	1.28
Stearic acid	2.64	2.09E-02	1.27
Docosahexaenoic acid	2.46	1.39E-02	1.28
Palmitic acid	2.26	4.08E-02	1.26
Eicosanoic acid	2.08	3.21E-02	1.14
1-Monopalmitin	1.95	1.24E-02	1.17
2-Monostearin	1.88	4.03E-02	1.03
Myristic acid	1.76	1.14E-02	1.22
2-Monopalmitin	1.73	4.23E-02	1.04
Arachidonic acid	1.69	4.03E-02	1.13
Oleic acid	1.59	4.60E-02	1.01

FC (fold change) was the ratio of the peak intensities of the metabolites between the thermal treatment group and control group on the 14th day. P-value was required from student's t-test. VIP (variable importance in projection) was identified by PLS-DA model.

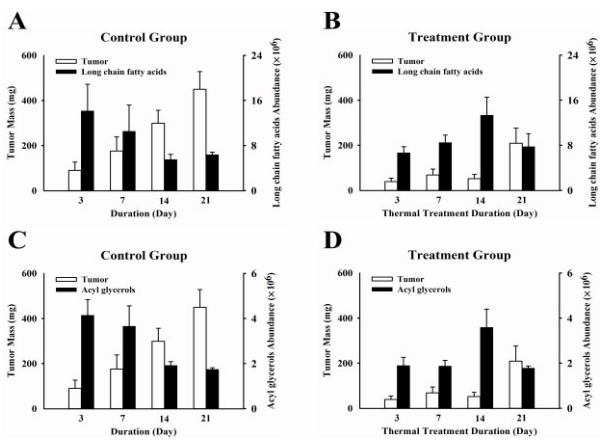

Figure 3. The correlation between tumor mass and total abundance of long chain fatty acids and acyl glycerols.

E. Discussion

We suggested that the breast tumors in the control group kept vigorous proliferating, and most long chain fatty acids were transferred to phospholipid membrane in order to support the rapid proliferation of tumor cells. For the thermal treatment group, however, the tumor cell proliferation was greatly inhibited in the first two weeks. There was an obvious accumulation of long chain fatty acids in tumor tissues, and some of them were synthesized to acyl glycerols and stored as energy. In the third week, due to the recovery of tumor cell proliferation, long chain fatty acids were again involved in the membrane synthesis, and acyl glycerols were also degraded into long chain fatty acids to supply as the sufficient source of long chain fatty acids (Fig. 4).

IV. CONCLUSION

In this study, a long-term mild hyperthermia at 39 °C was imposed on the mice breast tumors to investigate the effect of the thermal energy on tumor metabolism and growth. Results showed that long-term mild hyperthermia delayed tumor growth and affected tumor metabolism. GC-MS analysis indicated that there were significant differences in the abundance of the long chain fatty acids and acyl glycerols of thermally treated tumors compared to the tumors in the control. Based on the metabonomics results, we suggested that the tumor cell proliferation was restrained by the long-term mild hyperthermia, which contributed to the accumulation of long chain fatty acids and acyl glycerols in the first two weeks of treatment. However, the abundance of the long chain fatty acids and acyl glycerols declined in the control group in order to meet the rapid tumor cell proliferation. These observations suggested that the blockade of long chain fatty acid synthesis during mild hyperthermia treatment of tumors could improve the long-term treatment effect by limiting the supply of substance and energy for tumor re-growth.

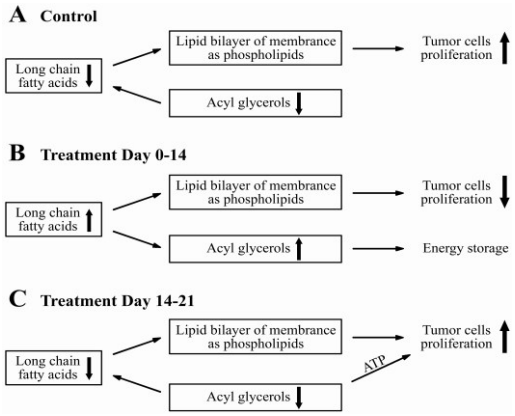

Figure 4. Interpretation of the observed experimental results

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