Formosanin C promotes the curative efficacy of ultrasound-guided radiofrequency ablation in a mouse model of breast cancer

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Received September 9, 2020; Accepted March 31, 2021

DOI: 10.3892/ol.2021.12811

Abstract. Breast cancer is the leading cause of tumor-associated death among women worldwide, and new therapeutic strategies are required to improve the post-surgery prognosis and quality of life of patients. Radiofrequency ablation (RFA) is a less invasive approach compared with traditional surgical resection to treat malignancies, and the combination of RFA and chemotherapeutic agents, including formosanin C (FC), can synergistically improve the curative effects against breast carcinoma. However, the detailed mechanisms remain unclear. In the present study, nude mice were used to identify the influence of FC on the therapeutic efficacy of RFA for breast cancer. Flow cytometry was performed to demonstrate the proportional alteration of CD8\textsuperscript{+} and CD45\textsuperscript{+} T cells with different biomarkers, including CD107a, IFN\textgamma and TNF\textalpha. It was demonstrated that FC enhanced the therapeutic efficacy of RFA in breast cancer, while RFA combined with FC improved the proportion of IFN\textgamma\textsuperscript{+} and TNF\textalpha\textsuperscript{+} CD8\textsuperscript{+} T cells and CD107a\textsuperscript{+} CD8\textsuperscript{+} T cells in tumor-infiltrating lymphocytes, thus increasing the immune responses caused by surgery and chemotherapy. The present study indicated that FC may promote the curative efficacy of ultrasound-guided RFA against breast tumor by regulating adaptive immune responses.

Introduction

Breast cancer has ranked as the second most common and the fifth most lethal malignancy in the world, with ~1.7 million women diagnosed with this disease in 2012 (1,2). The heterogeneity of breast tumors and their distinct prognosis require systemic therapeutic strategies and targeted treatment for different patients (3), making breast cancer one of the most challenging solid tumors to treat.

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Key words: radiofrequency ablation, formosanin C, breast cancer, CD8\textsuperscript{+} T cell

Materials and methods

Regents. FC used in the present study was purchased from Selleck Chemicals. Ethanol (Thermo Fisher Scientific, Inc.)
was used to dissolve FC, and FC solution was stored as -20°C for further experiments.

**Animals.** Adult BALB/c nude mice (female, 4-6 weeks; n=55) weighing 18-22 g were obtained from Charles River Laboratories, Inc. All mice used in the present study were kept in a virus/antigen-free system with permanent humidity and constant temperature (humidity, 40-70%; temperature, 20-26°C; 12 h light/dark cycle) and free access to food and water. Animal studies were approved by the Ethics Committee of Liaocheng People's Hospital (Liaocheng, China). MDA-MB-231 cells were purchased from Hunan Fenghui Biotechnology Co., Ltd., and 1x10⁶ cells dissolved in PBS buffer (Thermo Fisher Scientific, Inc.) were injected subcutaneously into the axilla of each nude mouse. When the tumors became palpable (after 1 week), the tumorigenic nude mice were divided into five treatment groups (n=8/group; the remaining 7 mice were not used as the tumor did not grow in time): Group I, control; Group II, 10 mg/kg FC; Group III, 20 mg/kg FC; Group IV, RFA; Group V, RFA+10 mg/kg FC; and Group VI, RFA+20 mg/kg FC. Isoflurane (2%) with oxygen was applied to induce the inhalant anesthesia of the mice, and RFA was only performed in the mice of Groups IV, V and VI. The same surgical procedures without radiofrequency heating were performed in the mice in Groups I, II and III. FC was injected intraperitoneally at the second day and the same volume of ethanol was used as the negative control. The tumor volumes of nude mice were monitored at days 0, 3, 6, 9, 12, 15, 18 and 21, and the animal health and behavior were monitored every three days. The total duration of animal experiments was 21 days. No mice died during the experiment. The maximum tumor diameter was 17 mm (not exceeding 20 mm), and then mice were sacrificed by cervical dislocation. The death of the mice was confirmed by the stop of the mouse thorax and cardiac arrest. The doses of FC administration were selected according to a previous study (16).

**Flow cytometry.** The TILs of each mouse were collected and made to single-cell suspension with cell staining buffer targeting IFNγ, TNFα, CD8, CD45 and CD107a (Thermo Fisher Scientific, Inc.) respectively. FC receptor blockers (Thermo Fisher Scientific, Inc.) were used to block non-specific effects on staining results. Fluorescently labeled antibodies (Abcam; CD8 (APC), cat. no. ab237368, 1:500; CD45 (FITC), cat. no. ab210225, 1:100; CD107a (Alexa Fluor® 488), cat. no. ab187591, 1:50; IFNγ (PE), cat. no. ab95673, 1:50; TNFα (Alexa Fluor 488), cat. no. ab237353, 1:50) were then incubated with the cell suspension at 4°C overnight, and 0.1% PBS buffer was used to wash the cells. A flow cytometer (Thermo 100022777; Thermo Fisher Scientific, Inc.) was used, and the results were analyzed by FlowJo software (Version 7.6.1; Treestar, Inc.). For intracellular cytokine staining, harvested cells were stimulated with PMA (10 ng/ml) and ionomycin (1 mg/ml) for 4 h and incubated for the last 1 h with brefeldin A (10 mg/ml) at 37°C. IFNγ- and TNFα-producing cells were examined by flow cytometry.

**Immunonephelometric assay.** The blood samples of mice were collected immediately after sacrifice and were centrifuged to obtain the serum (4°C, 4,000 x g, 15 min). All serum samples were kept at -80°C for further experiments. Levels of immunoglobulins, including IgG (cat. no. SNM259), IgM (cat. no. SNM260) and IgA (cat. no. SNM258), in mice serum were determined using an Immunoglobulin Assay kit (Beijing Biolab Technology Co., Ltd.) according to the manufacturer's protocol.

**Statistical analysis.** All experiments were repeated independently at least three times for the accuracy of the data, which were represented as the mean ± SD. GraphPad Prism v7.0 software (GraphPad Software, Inc.) was used to analyze the raw data and construct curves and histogram. One- or two-way ANOVA analysis, followed by Tukey's post-hoc test, was performed to analyze the data. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**FC enhances the therapeutic efficacy of RFA in breast cancer.** The molecular structure of FC used in the present study is shown in Fig. 1A; FC is a type of steroidal saponin with four sugars isolated from *Rhizoma paridis*. To demonstrate that the combined treatment of RFA and FC was able to improve the curative efficacy and prognosis, nude mice were used to establish the animal model. As shown in Fig. 1B, FC procedure alone significantly decreased the volume of breast tumors compared with that in the control group. However, the tumor volume still increased over time even though the nude mice received RFA treatment. On the other hand, the combination of RFA and 10 or 20 mg/kg FC treatment markedly inhibited the increase in tumor volume. Similarly, the administration of FC in combination with RFA significantly inhibited the tumor weight compared with RFA alone (Fig. 1C).

**Combination of FC and RFA improves the immune function of nude mice.** Previous studies have revealed the influence of FC on the immune responses of mice to tumor cells (13,17). Thus, the present study investigated the immunoglobulin levels in the serum of mice. Similar alterations in IgG, IgM and IgA levels under the influence of RFA and FC treatment were observed, as shown in Fig. 2A-C. Compared with that in mice in the control group, the immunoglobulin levels of mice in the RFA and FC groups were significantly increased, suggesting the antitumor functions of RFA and FC alone. Furthermore, the immunoglobulin levels of mice in the RFA + FC group were significantly higher than those in the other three groups. These findings indicated that the combination of RFA and FC significantly increased the immune function of nude mice by upregulating the immunoglobulin levels.

**Combination of FC and RFA increases the proportion of CD8⁺ T cells in TILs.** CD45⁺ and CD8⁺ T cells serve a crucial role in the cell-mediated immunity against cancer, and the proportion of CD45⁺ and CD8⁺ T cells in TILs generally determines the progression and prognosis of certain tumors (18). The present study investigated the influence of RFA and FC on the proportion of T-cell groups in TILs. As shown in Fig. 3A, either RFA or FC treatment alone increased the proportion of CD45⁺ and CD8⁺ T cells in TILs compared with the control group, and the combination of RFA and FC further increased their proportion.
With the combined treatment of RFA and FC, the proportion of CD45+CD8− T cells increased by 3-fold compared with the control group (Fig. 3B), and the proportion of CD45+CD8+ T cells increased by >5-fold (Fig. 3C).

**Combination of FC and RFA increases the percentage of IFNγ+ and TNFα+ CD8+ TILs.** IFNγ is a type of interferon secreted by T cells, and TNFα is a type of tumor necrosis factor secreted by monocytes and macrophages. They are both antitumor factors that participate in tumor destruction (19). The present study identified the percentage alteration of IFNγ+ and TNFα+ CD8+ TILs. As shown in Fig. 4A-C, the combination treatment of RFA and FC significantly increased the percentages of both IFNγ+ and TNFα+ CD8+ TILs compared with RFA alone, suggesting that this combined therapeutic strategy significantly improved the immune response of nude mice to the orthotic tumor.

CD107a is currently recognized as a marker protein for degranulation of cytotoxic T cells (20). Therefore, the present study investigated the influence of RFA and FC on the CD107a-expressing CD8+ TILs. As shown in Fig. 5A and B, the combined therapy of RFA and FC significantly increased CD107a-expressing CD8+ TILs compared with RFA alone, suggesting that the tumor destruction induced by cytotoxic T cells was elevated with the combined treatment of RFA and FC.

**Discussion**

The global incidence of breast cancer has been on the rise since the late 1970s, and ~1/8 women in the United States develops breast cancer in their life time (21). Thus, breast cancer has become a major public health issue in modern society (22). Breast cancer screening improves the proportion of early diagnosis, and new therapeutic strategies increase the curative efficacy, both of which contribute to the decreased mortality and increased prognosis of patients with breast carcinoma (23). As a result, the global breast cancer mortality rate has shown a downward trend since the 1990s (21). In the last two decades, the management of breast cancer has entered the era of comprehensive treatment, forming a treatment model that focuses both on local breast cancer treatment and systemic treatment. However, surgery, radiotherapy, chemotherapy, endocrine therapy, bio-targeted therapy and drug-assisted therapy performed in the treatment of breast tumor can lead to unbearable side effects that significantly impact the quality of life of patients (24). Additionally, the recurrence of breast tumor is a problem that cannot be ignored in the clinic, which
greatly impacts the prognosis of patients (25). The present study reported a new potential therapeutic strategy for breast carcinoma, which may improve the treatment efficacy and inhibit the relapse of breast tumor at the same time. The combination of RFA and FC treatment significantly inhibited the proliferation of remnant tumor compared with RFA alone or FC alone. Notably, it was revealed that FC treatment induced significant upregulation of immune functions in nude mice by improving their immunoglobulin levels. RFA treatment also seemed to significantly upregulate immunoglobulin levels. The adaptive immune response was markedly activated by the combination of RFA and FC.

Tumor RFA utilizes electrode needles inserted into the tumor to produce a radiofrequency current, which causes high-speed particle movement and friction inside the tumor tissues (26). The heat generated by the electrode needles results in high temperature, which conducts outward and induces the coagulation and shrinkage of solid tumors (27). RFA is an improved, minimally invasive method for treating tumors compared with other surgical procedures. RFA is able to ablate tumors of 5-7 cm in size and is particularly suitable for solid tumors of the liver and lung, with limited side effects and higher curative efficacy compared with traditional chemotherapies (28). In the last decade, the application of RFA for the treatment of breast carcinoma and related diseases has drawn great attention from clinicians. For instance, previous research has demonstrated that RFA can be used to treat liver metastasis from breast cancer, and the outcomes of RFA treatment are similar to hepatic resection, which is the traditional surgical procedure for this disease, with similar median survival times (41 vs. 37 months) and 3-year overall survival rates (55.4 vs. 52.6%) (29). However, the re-proliferation of the breast cancer cells located at the margins or clefts of overlapping ablation zones represent a big problem for RFA treatment of breast tumor (30). A possible way to solve this problem may be to improve the completeness of RFA treatment and enlarge the heating zones of these electrode needles, but the corresponding over-destruction of adjacent normal tissues can be unbearable for patients (31).

Previous studies have revealed that the combination of RFA and other chemical agents can effectively improve the treatment outcomes for a variety of tumors (32). For instance, Ahmed and Goldberg (33) demonstrated that liposomal doxorubicin combined with RFA treatment markedly improved the drug concentration in metastatic liver tumor tissues and improved the therapeutic outcomes in vivo. Wu et al (34) revealed that the combination of thermosensitive liposomal vinorelbine treatment and RFA procedure increased the end-point survival of mice with liver tumor. Similarly, the present study revealed that the combination of RFA procedure and FC significantly improved the treatment efficacy in a breast cancer mouse model. The combined

Figure 3. Combination of FC and RFA increases the proportion of CD8+ killer T cells in TILs. (A) Tumors were resected and digested to generate single-cell suspension. Representative flow cytometric plots showing CD8+ and CD45+ T cells in single-cell suspension after FC or RFA treatment. (B) Percentages of CD45+ CD8+ cells within single-cell suspension. (C) Percentages of CD45+CD8+ TILs. Error bars indicate SEM; P-values were calculated using ANOVA with Tukey’s test. **P<0.01. FC, formosanin C; RFA, radiofrequency ablation; TIL, tumor-infiltrating lymphocyte.
Figure 4. Combination of FC and RFA increases the percentage of IFNγ⁺ and TNFα⁺ CD8⁺ TILs. (A) Representative flow cytometric plots of IFNγ⁺ and TNFα⁺-expressing CD8⁺ TILs of nude mice from control, RFA, FC and RFA+FC groups. Percentages of (B) IFNγ⁺ and (C) TNFα⁺ within CD8⁺ TILs of nude mice from control, RFA, FC and RFA+FC groups. Error bars indicate SEM; P-values were calculated using ANOVA with Tukey's test. **P<0.01. FC, formosanin C; RFA, radiofrequency ablation; TIL, tumor-infiltrating lymphocyte; SSC, side scatter.

Figure 5. Combination of FC and RFA increases the percentage of CD107a⁺ CD8⁺ TILs. (A) Representative flow cytometric plots of CD107a⁺-expressing CD8⁺ TILs of nude mice from control, RFA, FC and RFA+FC groups. (B) Percentages of CD107a⁺ within CD8⁺ TILs of nude mice from control, RFA, FC and RFA+FC groups. Error bars indicate SEM; P-values were calculated using ANOVA with Tukey's test. **P<0.01. FC, formosanin C; RFA, radiofrequency ablation; TIL, tumor-infiltrating lymphocyte; SSC, side scatter.
therapy effectively inhibited the increase of the volume of orthotopic breast tumors. Another study has demonstrated that intratumorally injected paclitaxel improves the treatment outcomes of RFA in breast cancer and inhibits the toxicity of paclitaxel to normal tissues, compared with oral or intravenous administration of the agent (34). Thus, a more detailed and accurate mode of drug administration to improve chemotherapy efficacy and decrease the toxicity for the current combined therapy of RFA and FC should be further investigated in the future (34).

FC is a diosgenin isolated from Rhizoma paridis and is an important active ingredient of some traditional Chinese medicine with anticancer, anti-inflammatory and anti-venom functions (17). In recent years, the anticancer function of FC has drawn great attention, and accumulating evidence has demonstrated that FC can mediate the body immune responses against tumor cells and thus inhibit the progression of liver and lung cancer (13,17,35). For instance, the synergistic anticancer function of FC and polyphyllin I in liver cancer has been revealed (10). It has been reported that FC can inhibit the pulmonary metastasis of lung carcinoma by suppressing matrix metalloproteinases (10). In the present study, it was reported that FC treatment alone inhibited the growth of the orthotopic breast tumors in nude mice and 20 mg/kg FC administration significantly suppressed the increase of tumor volume in nude mice. The combination of FC and RFA treatment dramatically improved the adaptive immune response of nude mice and altered the proportion of different cells in the TILs, thus inhibiting the relapse of orthotopic tumor.

CD8 co-receptors are mainly expressed on the surface of cytotoxic T cells, natural killer cells, cortical thymocytes and dendritic cells (36). CD8+ T cells serve an important role in tumor destruction (15,37). CD45 co-receptors are expressed on all leukocytes and are known as common leukocyte antigens, and they are key molecules for signal transduction on the cell membrane. CD45 serves a crucial role in lymphocyte development, maturation, functional regulation and signal transmission. According to the type of CD45 molecules expressed by T cells, human T cells can be divided into CD45RA+ initial T cells and CD45RO+ memory T cells (38). CD107a co-receptors participate in the antitumor, antiviral infection and immune regulatory functions of natural killer cells (39,40). In the present study, it was revealed that the combination of RFA and FC increased the proportion of CD8+, CD45+ and CD107a+ T cells in the TILs of orthotopic breast tumors in nude mice, suggesting that this therapy significantly improved the T-cell-related tumor destruction in the current mouse model.

In conclusion, the present study reported the synergistic anticancer function of RFA procedure and FC administration for breast cancer in vivo. It was identified that RFA combined with FC improved the proportion of IFNγ+ and TNFα+ CD8+ T cells and CD107a+ CD8+ T cells in TILs of orthotopic breast tumors in nude mice, and thus increased the immune responses caused by surgery and chemotherapy. As a result, FC promoted the curative efficacy of ultrasound-guided RFA in breast tumor by regulating adaptive immune responses. The current study may provide a potential therapeutic strategy for breast cancer in the clinic.

Acknowledgements
Not applicable.

Funding
The present study was supported by the Natural Science Foundation of Shandong Province (grant no. bs2015sw016).

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
Conceptualization: ZC and JL; performed experiments and analyzed data: ZC, JL, QC, FL and GZ; funding acquisition: ZC; project administration: ZC and JL; supervision: ZC and JL; validation: ZC, JL, QC, FL and GZ; writing-original draft: ZC, JL, QC, FL and GZ; writing-review & editing: ZC, JL, QC, FL and GZ. All authors have read and approved the manuscript. All authors confirm the authenticity of all the raw data.

Ethics approval and consent to participate
Animal studies were approved by the Ethics Committee of Liaocheng People’s Hospital (Liaocheng, China).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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