

ORIGINAL RESEARCH

Tumor growth inhibition and tumor specific immunity enhancement in endometrial carcinoma by high intensity focused ultrasound

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Abstract

Endometrial carcinoma (EC) is a type of epithelial malignancy prevalent in about 8% of the total malignancies in women. Development of new treatment methods is vital for improving the prognosis of endometrial cancer. High Intensity Focused Ultrasound (HIFU) is a safe method for oncological treatment with low complication rate. However, EC treatment by HIFU is rarely reported and its mechanism is unclear. Herein, the effects of HIFU on EC progression were detected. Data confirmed the inhibition of EC tumor growth in mice by HIFU. Moreover, HIFU inhibited the EC tumor apoptosis. It down-regulated the Treg cell production and enhanced the tumor-specific cytotoxicity. It restrained the Janus kinase/Signal transducer and activator of transcription 3 (JAK/STAT3) pathway *in vivo*, and suppressed the EC progression. HIFU could thus be a promising treatment for EC.

Keywords

Endometrial carcinoma (EC); High Intensity Focused Ultrasound (HIFU); Treg cell; Apoptosis; JAK/STAT3 pathway

1. Introduction

Endometrial carcinoma (EC) is an epithelial malignancy found in ~8% of all malignancies in women [1]. The female obesity rate has increased in recent years because of improved living standards, and high incidence of hypertension and diabetes mellitus, *etc.* The incidence of endometrial cancer is thus also on the rise [2]. EC can be categorized into four stages. The methods of examination include imaging such as ultrasound, magnetic resonance, *etc.*, and histopathology like uterine microbioscopy, curettage, *etc.* The surgical pathology staging is still used wherein the true staging of EC is determined according to the postoperative pathology. The prognosis of early EC patients is good with 5-year survival rate of 95%, while the 5-year survival rate is low with poor prognosis [3–5]. So, the prognosis of EC can be improved by finding new treatment methods.

High Intensity Focused Ultrasound (HIFU) is a safe treatment method because of its non-ionizing and non-invasive characteristics [6]. Studies show that HIFU is an oncological treatment which is repeatable with low complications [7]. In breast cancer, the HIFU combined with nanoparticles improves drug delivery and minimizes the side effects of treating tumors by drugs [8]. HIFU enhances the viability and apoptosis of breast cancer cells as inhibited by the paclitaxel *in vitro* and *in vivo*, and improves the animals' survival rate [9]. HIFU is beneficial for the early stage of EC. The indications and contraindications should be controlled in HIFU treatment. HIFU

therapy of prostate tumors increases cytotoxic T cells in spleen and tumor draining lymph nodes (TDLN) by downregulating the intratumor STAT3 activity. Furthermore, it increases the number and activity of dendritic cells and inhibits Treg cells generation. Resultantly, it increases anti-tumor response and inhibits distant tumor metastasis [10]. HIFU in EC thus affects the cancer progression with similar mechanism.

HIFU causes upregulation of spleen and lymph node natural killer (NK) cells, and circulating interleukin-2 (IL-2), and Interferon gamma (IFN-gamma) in neuroblastoma, triggering the immune sensitivity of refractory mouse neuroblastoma by checkpoint inhibitor therapy [11]. HIFU can thus regulate the immune microenvironment of tumors, and has anti-cancer therapeutic effects [12]. However, EC treatment by HIFU is rarely reported and the mechanism is unclear.

Herein, it was found that HIFU therapy in EC xenograft tumor models could have anti-tumor role by inhibiting the JAK/STAT3 pathway, inhibiting the Treg cells generation, and enhancing the tumor-specific cytotoxicity. HIFU could thus be a new treatment of EC.

2. Materials and methods

2.1 Tumor growth *in vivo* assay

Cells were injected to the nude mice. The tumor volume was measured every 7th day of the treatment. After 35 days, the tumors were analyzed for the control and HIFU treated groups.

2.2 HIFU treatment

Real-time ultrasound guided about the HIFU treatment and monitored treatment effects. The therapeutic transducer was calibrated by determining the radiation force in degassed water and had focal intensity of 10,000 W/cm² with maximum output power of 300 W. The animals were anesthetized and kept in lateral position to clearly observe the tumor with ultrasound. The skin surface was in close contact with the tank. The samples were imaged by the B-mode ultrasound. Point-by-point treatment of hypochoic tumor tissue was made at the treatment depth of 5 mm as the tumor was small, and the level of treatment was in one layer. The interval between treatment points was 1 mm, and 10 s for HIFU-10s group or 20 s for HIFU-20s group. Each group of mice was observed for 4 weeks after the treatment and killed.

2.3 Immunoblot assay

Total proteins were extracted from the samples, separated by Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), transferred to polyvinylidene fluoride (PVDF) membranes, and blocked using 5% milk. PVDF membranes were incubated with primary antibodies targeting the anti-Bax antibody (1:500, ab32503, abcam), anti-Bcl-2 antibody (1:500, ab182858, abcam), anti-cleaved-Caspase-3 antibody (1:1000, ab32042, abcam), anti-JAK2 antibody (1:500, ab108596, abcam), anti-p-JAK2 antibody (1:1000, ab32101, abcam), anti-STAT3 antibody (1:500, ab68153, abcam), anti-p-STAT3 antibody (1:500, ab267373, abcam), and anti- β -actin antibody (1:3000, ab8226, abcam) for 2 h, as well as with the secondary antibodies for 1 h. ECL kit was employed to detect the blots in membranes.

2.4 Immunohistochemical (IHC) and TUNEL assays

The sections were fixed by 4% paraformaldehyde (PFA) for 30 min and blocked by 2% bovine serum albumin (BSA). They were then incubated with antibodies for 2 h and with secondary antibodies for 1 h. TUNEL System Kit (G3250, Promega, Madison, WI, USA) was used for the TUNEL staining.

2.5 H&E staining

The tissues were sliced, and dehydrated and rehydrated in absolute alcohol. Slides were stained for 4 min with hematoxylin, rinsed, differentiated in 70% alcohol, counterstained with eosin Y, and cleared in xylenes before mounting.

2.6 Immunostaining

Tissue slices were mounted onto glass coverslips and stained for the proteins. AxioVision 8.0 software quantified the fluorescence of 20 cells per field in five random fields.

2.7 Flow cytometry assays

CD8⁺ cells were detected (Sigma Aldrich, USA). Cells were digested and mixed for 5 min in reaction buffer containing CD8 antibody. Cell proportions were analyzed by flow cytometer (Lyric, BD Biosciences, NJ, USA).

2.8 Statistics

Statistical analyses were made by using GraphPad 6.0 (National Institutes of Health, Bethesda, Rockville, MD, USA). Data were presented as mean \pm Standard deviation (SD). Student's *t*-test was employed for the comparisons. **p* < 0.05 was considered as significant.

3. Results

3.1 HIFU inhibiting the EC tumor growth in mice

Cells (10⁵) were subcutaneously injected to detect whether HIFU inhibited the EC tumor growth in mice. The nude mice were divided into 3 groups. HIFU treatment was given after 2 weeks of cells injection. The interval between treatment points was 10 s (HIFU-10s group) or 20 s (HIFU-20s group). The tumor volume was assessed on every 7th day till 35 days. HIFU treatment had restrained the EC growth with decreased tumor volume and weight (Fig. 1A, **Supplementary Tables 1 and 2**). H&E staining also indicated the inhibition of tumor growth in mice (Fig. 1B).

3.2 HIFU stimulating the EC tumor apoptosis in mice

Previously, it was found that HIFU treatment suppressed the EC growth in mice. It was then clarified if HIFU affected the EC cells apoptosis in mice. It was noticed through TUNEL assays that HIFU treatment contributed to EC cells apoptosis in tumor tissues of mice (Fig. 2A). Immunoblot assays revealed that HIFU treatment increased the cleaved caspase-3 and Bax, and decreased Bcl-2 in mice tumor tissues (Fig. 2B).

3.3 HIFU inhibiting Treg cell production and enhancing tumor-specific cytotoxicity in mice

Immunostaining assays depicted that HIFU treatment suppressed the expressions of CD4 and Foxp3 in mice tumor tissues, *i.e.*, the inhibition of Treg cell production (Fig. 3A,B). Similarly, FCM assays reflected that the CD8⁺ cells percentage was decreased upon HIFU treatment (Fig. 3C). HIFU thus inhibited the Treg cell production and enhanced the tumor-specific cytotoxicity in mice.

3.4 HIFU inhibiting the JAK/STAT3 pathway of EC tumor *in vivo*

The mechanism of HIFU suppressing the EC progression was detected. The immunoblot assays exhibited that HIFU treatment suppressed the phosphorylation levels of JAK2 and STAT3 in EC tumors from mice, *i.e.*, the inhibition of JAK/STAT3 pathway (Fig. 4).

4. Discussion

EC is a uterine body cancer referring to the endometrium cancer wherein the majority are adenocarcinomas [13]. It is among the three common malignant tumors of female reproductive tract. The treatment methods of EC include surgery, radiother-

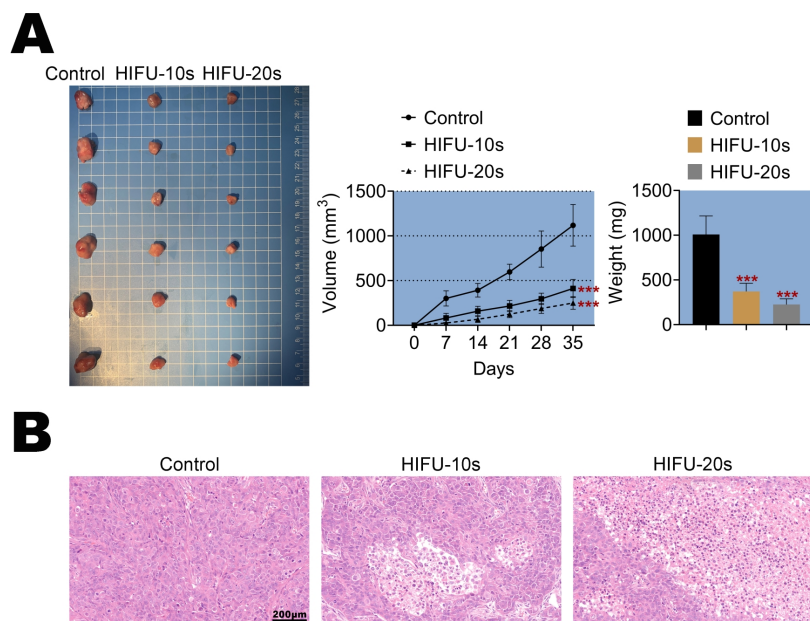


FIGURE 1. HIFU inhibited the EC tumors growth in mice. (A) HEC1B cells were subcutaneously injected into the nude mice abdomen ($n = 6$ for each group) to induce tumor growth. Tumor images were shown on left, and tumor volume and weight on right. (B) H&E staining exhibited the tumor tissues from mice in the indicated groups. Scale bar indicated $100 \mu\text{m}$. Data were presented as mean \pm SD. $***p < 0.001$ compared to the control. HIFU: High Intensity Focused Ultrasound.

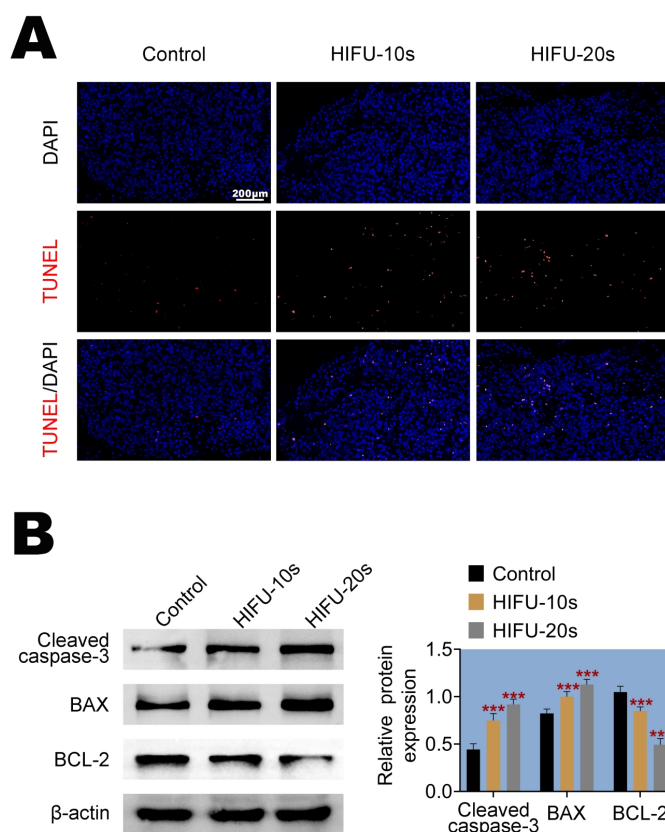


FIGURE 2. HIFU stimulated the EC tumors apoptosis in mice. (A) TUNEL assays revealed the degree of apoptosis in tumor tissues of mice with the indicated treatment. Scale bar, $200 \mu\text{m}$. (B) Immunoblot assays showed the expressions of Bax, Bcl-2, and cleaved caspase-3 in mice tumor tissues with indicated treatment. The quantification of expression level was shown. Data were presented as mean \pm SD. $***p < 0.001$ compared to the control. HIFU: High Intensity Focused Ultrasound; TUNEL: TdT-mediated dUTP Nick-End Labeling; DAPI: 4', 6-diaminyl-2-phenylindole; BCL-2: B-cell lymphoma-2.

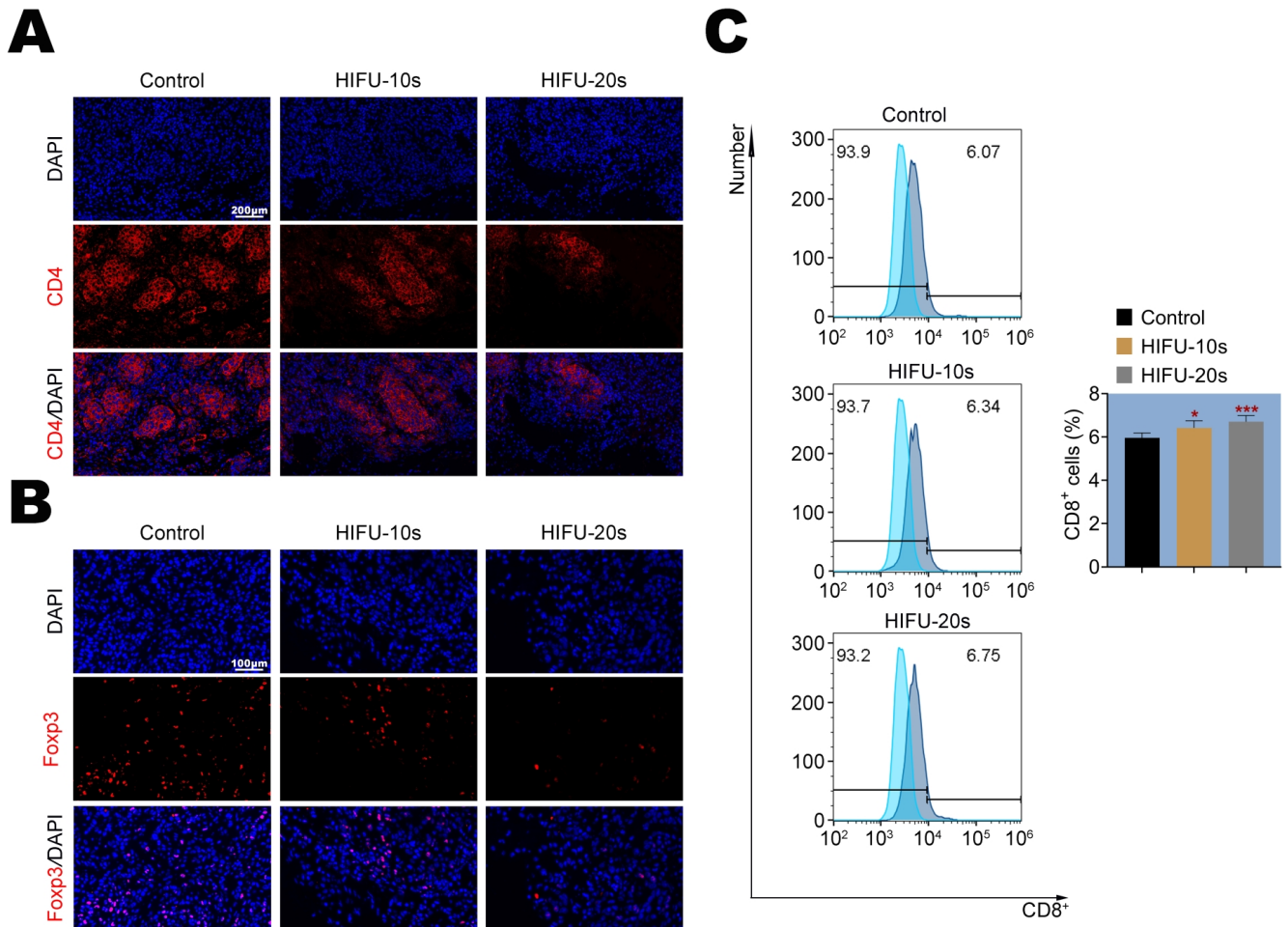


FIGURE 3. HIFU inhibited Treg cell production and enhanced tumor-specific cytotoxicity in mice. (A) Immunostaining depicted the CD4 expression in mice tumor tissues with the indicated treatment. Scale bar, 200 μm . (B) Immunostaining showed FOXP3 expression in mice tumor tissues with the indicated treatment. Scale bar, 100 μm . (C) FCM assays exhibited the CD8⁺ cells percentage in mice tumor tissues with the indicated treatment. Data were presented as mean \pm SD. * $p < 0.05$, *** $p < 0.001$ compared to the control. HIFU: High Intensity Focused Ultrasound; DAPI: DAPI: 4', 6-diaminyl-2-phenylindole; CD: cluster of differentiation; FOXP3: Forkhead box protein P3.

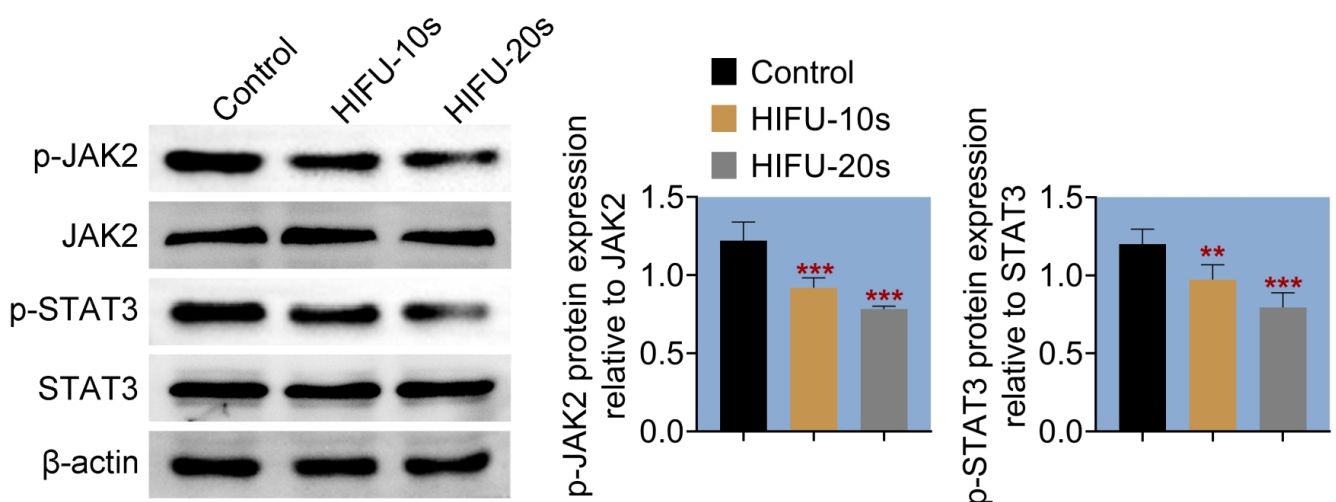


FIGURE 4. HIFU inhibited the JAK/STAT3 pathway of EC tumors *in vivo*. Immunoblot assays showed the expression and phosphorylation levels of JAK2 and STAT3 in mice tumor tissues with indicated treatment. The expression level quantification was shown. Data were presented as mean \pm SD. ** $p < 0.01$, *** $p < 0.001$ compared to the control. HIFU: High Intensity Focused Ultrasound; JAK: Janus kinase; STAT3: Signal transducer and activator of transcription 3.

apy, chemical anti-cancer drugs, and hormone therapy [13]. Surgery is preferred to treat EC because it is not much sensitive to radiotherapy, however long-term treatment is required after the surgery [13]. Studies believe that radiotherapy is only used for elderly or advanced patients having serious medical complications which cannot be operated [1]. EC is not much affected by anti-cancer drugs, and most are used as treatment for advanced EC or recurrent cancer [1]. Chemotherapy for EC is mainly the single drug at early stage. Recently, the targeted drugs such as bevacizumab, sorafenib and sunitinib are developed with certain efficacy in treating EC [14]. More effective treatment methods are needed to combat this disease. Herein, it is depicted that HIFU can efficiently and reproducibly treat EC with low complication rate.

HIFU is a new tumor therapy [7]. In this method of treating tumor, the ultrasound localization kills tumor cells in the target area without damaging the surrounding normal tissue. So, HIFU is a minimally invasive treatment of tumor tissue [15]. Compared to the other tumor treatment methods, HIFU treatment does not require surgery, no bleeding, faster recovery and low treatment cost. This method can activate the body's immune system, however it does not involve radiations and chemical damage [16]. HIFU has been suitable for treating solid tumors in tissues and organs including liver, bone, breast, pancreas, prostate, kidney, soft tissue, uterine fibroids, adenomyosis, benign prostatic hyperplasia, and retroperitoneal or abdominal pelvis [7]. Herein, HIFU suppresses EC progression in mice as found by constructing *in vivo* tumor growth model of EC.

HIFU can mediate the tumor immune microenvironment [17]. HIFU therapy in prostate tumors increases cytotoxic T cells in spleen and down-regulates intratumor STAT3 activity, while inhibits Treg cells generation, thereby increasing the anti-tumor response and inhibiting the distant tumor metastasis [18]. HIFU upregulates NK cells and circulating IL-2, IFN- γ and dAMP in neuroblastoma. It triggers the immune sensitivity of refractory mouse neuroblastoma towards checkpoint inhibitor therapy [19]. It is found herein that HIFU therapy inhibits Treg cell generation and enhances tumor-specific cytotoxicity by inhibiting the JAK/STAT3 pathway activation. This study suggests that the effect of HIFU on tumor immunity may further influence the EC progression. Multiple drugs or proteins have an impact on EC progression through JAK/STAT3 pathway. It is also found that HIFU affects EC *via* a similar pathway [20]. Moreover, multiple mechanisms are involved in the EC progression caused by HIFU treatment, which need further study [21].

5. Conclusions

In summary, HIFU treatment inhibits Treg cell generation, enhances tumor-specific cytotoxicity by inhibiting JAK/STAT3 pathway, and thus suppresses the EC progression.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

SHY and XWF—designed the study and carried them out, prepare the manuscript for publication and reviewed the draft of the manuscript. SHY, PG, WKH and FL—supervised the data collection, analyzed the data, interpreted the data. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical approval was obtained from the Ethics Committee of Cancer Hospital Affiliated to Xinjiang Medical University (Approval no. K-2022016). Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found, in the online version, at <https://oss.ejgo.net/files/article/1713791784513880064/attachment/Supplementary%20material.docx>.

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