Original Article Pathologic evaluation of uterine fibroids ablated with high intensity focused ultrasound

Xin Xiong¹, Fei Chen^{2,3}, Jinyun Chen³, Ryan S Hsi⁴, Jingxian Wu⁵, Jianzhong Zou³, Xuemei He³

¹College of Medical Informatics, Chongqing Medical University, Chongqing, China; ²Department of Ultrasound, The Second Affiliated Hospital of Guangzhou Medical University, Guangzhou, China; ³College of Biomedical Engineering, Institute of Ultrasound Engineering in Medicine, Chongqing Key Laboratory of Biomedical Engineering, Chongqing Medical University, Chongqing, China; ⁴Department of Urology, University of Washington School of Medicine, Seattle, USA; ⁵Department of Pathology, Chongqing Medical University, Chongqing 400016, China

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Abstract: Purpose: The aim of this study was to evaluate the pathologic changes including estrogen receptor (ER) and progesterone receptor (PR) expression of the uterine fibroid specimens treated by ultrasound guided high intensity focused ultrasound (HIFU) ablation. Materials and methods: Twenty post-hysterectomy specimens with uterine fibroids were ablated by ultrasound guided HIFU. Pathological evaluation was performed with 2,3,5-triphenyltetrazolium chloride (TTC), hematoxylin-eosin (H&E), and immunohistochemical staining for ER and PR expression. Results: The margin between the targeted and the non-targeted area was clearly demarcated on TTC staining. The targeted treatment area demonstrated coagulation necrosis on H&E staining. ER and PR expression in the fibroid tissue were both completely lost in the targeted region. A transition region consisting of several layers of ER and PR positive cells at the periphery of the treated area was identified. Conclusion: In addition to H&E staining, post-treatment ER and PR status may be an indicator of HIFU effectiveness.

Keywords: Focused ultrasound, ablation, uterine fibroid, estrogen receptor, progesterone receptor

Introduction

Uterine fibroids are associated with menorrhagia, pelvic pain, dyspareunia, infertility, and complications during pregnancy. They represent the most common female reproductive tract tumor and occur in 20-40% of reproductive-aged women [1-3]. In recent years, with the shifts in cultural attitudes and the productive age rising, women are increasingly willing to be treated noninvasively for these benign diseases [4].

High intensity focused ultrasound (HIFU) therapy has been described as a minimally-invasive technique used to provide local control of uterine fibroids while allowing for uterine preservation with side effects being uncommon [5-9]. Current therapy has focused on intra-tumor treatment with the goal of improving clinical symptoms and shrinking tumor size [10]. According to early reported clinical trials with magnetic resonance guided focused ultrasound (MRgFUS) therapy, an average of 10% to 50% tumor volume was treated with 70% to 90% symptomatic improvement in 6 months. However, up to 30% of patients sought alternative treatment of fibroid symptoms at 12 months [10]. More recently, up to 90% tumor volumes have been treated with HIFU therapy with 82% tumor volume shrinkage at 6 months follow-up; about 4 of 76 (5.2%) patients receiving repeat HIFU therapy for enlarging residual tumor [11]. The reintervention rate is 14% to 22% (depending on the different type of uterus myoma image intensity) within 24 months after HIFU therapy [12].

Uterine fibroid growth and development are hormone dependent processes. Animal and human studies have shown that high expression of ER and PR in uterine tissue is associated with uterine leiomyoma growth. Both ER and PR are expressed at higher levels in fibroids than in the myometrium under various endocrine conditions. Although the finding remains controver-

sial, we believe evolving evidence generally supports the concept that ER and PR are often higher in fibroids than in surrounding myometrium [13-17]. Therefore determining ER and PR expression in uterine fibroid tumors treated with HIFU could be good indicators of evaluating treatment effectiveness and potentially be useful clinically to predict aggressive tumor regrowth. To our knowledge, little research related to pathology, ER and PR expression of human uterus fibroids treated with HIFU has been reported. The purpose of this study was to investigate the pathologic changes including the status of ER and PR expression in uterine fibroids after HIFU treatment with an ex vivo model.

Materials and methods

Patients

From October 2008 to March 2009, 35 consecutive patients undergoing hysterectomy were included in this study. Preoperative evaluation was performed by two gynecologists using available clinical and radiologic data. Five patients with suspected malignant uterine disease, including endometrial cancer, uterine cervix cancer, and sarcomatoid lesions, were excluded. In the 30 remaining patients, ten patients were excluded for adenomyosis, leaving 20 hysterectomy specimens for pathological review. In the 20 hysterectomy specimens, all of them were multiple uterine myomas. The size range of the uterine fibroids was from 3.0-10.8 cm in diameter. The average uterine fibroids diameter was 6.85±2.65 cm × 5.71±1.95 cm × 5.21±1.56 cm based on pretreatment ultrasound measurements. Only intramural myomas were chosen as targets. The study was performed after approval by a local ethics committee in accordance with the specification stipulated by the Helsinki Committee. Each patient signed written informed consent at study inclusion.

HIFU therapy

Fresh hysterectomy specimens were placed in cooled 0.9% normal saline and degassed for 25 minutes. The degassed tissue was fixed in a treatment chamber with a treatment window for the HIFU device as previously described [18]. The chamber was placed into a water tank filled with degassed water with the air concentration at less than 3 ppm.

A Model JC-200 HIFU system [Chongqing Haifu (HIFU) Tech Co, Ltd, Chongging, China] was used to deliver focused ultrasound to the fibroid tumors. This system was the same as is used in our clinic. The technical details of this device have been previously described [18-21]. The device is guided by real-time ultrasound imaging during the treatment. It operates at a frequency of 1.6 MHz with focal length at 90 mm. The focal region is 3.3 mm along the beam axis and 1.1 mm in the transverse direction. The maximum focal intensity is 25000 W/cm². Using a fixed-point ablation technique, homogeneous regions within each fibroid were ablated with acoustic power set at 300 W and at therapeutic depths ranging from 2-4 cm depending on the size of tumor. The interval between every two targeted regions was 2 cm. For each targeted area, treatment duration was 20 to 40 s (average 25±7 s), and was stopped when a hyperechoic area was visualized on grey-scale imaging. Grey-scale changes in the targeted tumor have been reported to correlate well with HIFU-induced necrosis [19].

Histological examination

After treatment, the targeted area with a surrounding 10 mm tissue margin was sent for pathologic investigation. Specimens were serially sectioned into 1 mm-thick slices. The sectioned tissue was incubated for 30 minutes in 2% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, St. Louis, MO) solution at room temperature (18-20°C) to allow determination of tissue viability. The remaining tissue was fixed in 10% phosphate-buffered formalin and embedded in paraffin. Six consecutive 4-µm thick slices were cut from each paraffin block. Of these, one slice was stained with H&E staining, two slides for ER protein evaluation, two slides for PR protein evaluation and one slide for Negative Control Reagent. The cocktail of mouse IgG was used as negative control reagent. Immunohistochemistry controls were provided in the kit. Two positive control slides containing formalin fixed, paraffin-embedded cells representing moderate levels of ER/PR protein expression were available in the kits.

An ER/PR diagnostic kit (Maixin Bio Co, Fuzhou, China) was used. In brief, after specimen's



Figure 1. Gross specimen of uterine fibroid. A. The area between two arrows is the targeted region, without clear border observed by naked eyes. B. With TTC staining, the non-targeted region was stained red with clear border.



Figure 2. Microscopic view $(40 \times)$ of the transition zone of HIFU treatment on a uterine fibroid specimen. Typical appearance of H&E staining of the targeted region $(40 \times)$, which appears darker than the non-targeted region from H&E staining. The border is sharply demarcated (arrow).

deparaffinization and rehydration, the main kit procedures were as follows: (1). Epitope Retrieval in pressure cooker. Incubate 5 minutes at 125°C; (2). Incubation (5 min) with ER/PR kit peroxidase-blocking reagent; (3). Incubation (30 min) with the primary antibody. Rat antihuman estrogen receptor antibody was added at a concentration of 1:100; (4). Thirty minutes incubation with ER/PR kit Visualization Reagent; (5). Ten minutes incubation with ER/PR kit DAB + substrate chromogen. The samples were then coverslipped using an aqueous mounting medium for pathologic investigation [22].

Evaluation and analysis

TTC, H&E and immunohistochemical staining were evaluated qualitatively to analyze the effect of HIFU therapy on uterine fibroids lesions. For the determination of ER and PR expression, complete absence of expression in the targeted region was recorded as negative, and any visible immunoreaction of the tumor cells was considered to be positive for antigen expression. Rates of ER and PR positive expression in the targeted, non-targeted and transitional region were calculated and compared. The definition of positive rate was the number of positive ER/PR expression cells divided by the number of the whole cells in one visual field under 400X magnification. From each sample, four fields of interest were chosen, and the average positive rate of ER/PR was calculated. According to the number of positive cells, the rank was defined as follows: Scale 0: number of positive cells was less than 10%, Scale I: 11%~25%; Scale II: 26%~50%; Scale III: >50%.

Rank test was used to compare the status of ER and PR expression in the transitional region and non-targeted region. P<0.01 was defined as significantly meaningful.

Results

Gross histopathologic changes and TTC staining

On gross exam, the HIFU targeted region is difficult to discern by appearance only (Figure 1A),





Figure 3. ER immunohistochemical staining in uterine fibroid tissue treated by HIFU. A. ER immunohistological staining (40^{\times}), mainly showed the HIFU targeted region (non-arrow pointed region). B. ER immunohistological staining (40^{\times}), mainly showed the transitional border of the HIFU targeted region (arrow), layers of ER positive cells and negative cells were seen in this region. C. ER immunohistological staining (20^{\times}), mainly showed the strong positive brown staining (arrow) in non-targeted region, the transitional region and the targeted region can be seen.

but the treated tissue was slightly firmer than the surrounding non-treated region upon palpation. With TTC staining, viable tissue was stained in red while the necrotic region did not stain, consistent with necrotic changes (**Figure 1B**).

H&E staining

Generally, viable uterine fibroids tumor specimens were mainly composed of smooth muscle cells and fibrous connective tissue. Smooth muscle cells appeared uniformly-sized spindle shaped with rhabditiform nuclei. Cells were arranged in a swirl-type pattern. In this study, the characteristics of coagulation necrosis caused by HIFU were identified by pyknotic changes in the nuclei and cytoplasm concentration. Both basophilic nuclei and eosinophilic cytoplasm in tumor cells were visualized in the HIFU targeted region, while the cytoarchitecture of the targeted region appeared preserved (**Figure 2**).

ER and PR immunohistochemical staining

In the targeted region, ER and PR expression were both completely lost (**Figures 3A**, **4A**). The demarcation between the targeted and nontargeted region was clear. A transitional region with a width of 5 to 8 layers of cells with scattered positive expression of ER and PR was observed (**Figures 3B**, **4B**). The scale rates of both positive ER and PR in the transitional zone of all 20 samples were less than 10% (Scale 0). In the non-targeted region around the targeted region (**Figures 3C**, **4C**), for the ER, the positive scale rates were 3 of Scale II and 17 of Scale III. For the PR positive rate, 5 of Scale II and 15 of Scale III were detected. The Scale rank of posi-





Figure 4. PR immunohistochemical staining in uterine fibroid tissue treated by HIFU. A. PR immunohistological staining ($40\times$), mainly showed the HIFU targeted region (non-arrow pointed region). B. PR immunohistological staining ($40\times$), mainly showed the transitional border of the HIFU targeted region (arrow), layers of PR positive cells and negative cells were seen in this region. C. PR immunohistological staining ($20\times$), mainly showed the strong positive brown staining (arrow) in non-targeted region, the transitional region and the targeted region can be seen.

tive ER/PR expression in the transitional zone is significantly lower than that of the non-targeted region (P<0.01).

Discussion

The assessment of cell viability at the margins of the HIFU targeted zone may be important to determine treatment effectiveness in tumor ablation. Previous animal studies and clinical trials have used the H&E staining to judge the viability of cells after ultrasound ablation [19, 23-26]. The typical characteristics of necrosis include pyknotic nuclei, karyorrhexis, and karyolysis, which are indicators of lethal and irreversible cell damage. In the current study, the predominant post-treatment changes in the uterine fibroids were pyknotic nuclei and cytoplasm concentration. The boundary between the targeted and non-targeted regions after HIFU treatment can be differentiated on H&E staining by the density of staining, because the necrotic tissue maintains its cytoarchitecture with the surrounding tissue. However, according to some authors, the staining characteristics of the nuclear chromatin within the HIFU targeted area can appear preserved, even in necrotic areas due to fast thermo-coagulation [18, 20]. Moreover, coagulated necrosis has been verified in these cells under further electron microscopy and NADH staining [18, 20, 27]. In this study, karyorrhexis and karyolysis were rarely observed in the targeted region. Therefore, under these criteria, when the appearance of a coagulated region appeared similar to viable cells, it is hard to determine treatment effectiveness after HIFU therapy using H&E staining.

TTC staining on normal tissue stains red when the tissue contains active dehydrogenase, which is an indicator of cell viability, while a negative result indicates the irreversible cellular damage [27, 28]. In this study, the boundary between the viable and necrotic region appeared sharply marginated under gross exam. Therefore, TTC can be used as an indicator of coagulation necrosis during early stages of treatment with HIFU. However, TTC staining is not practical clinically due to the large sample size requirement for staining. Immunohistochemical stain is specific reaction between antigen and antibody. The high temperature caused by HIFU will induce the protein denaturation; theoretically, the antigen including ER/PR will become undetectable and lose their function. Wu et al. found that no expression of PCNA, MMP-9, and CD44v6 was detected within the treated tumor cells in the HIFU group with immunohistochemical staining, indicating that the treated tumor cells lost the abilities of proliferation, invasion, and metastasis [29].

As a hormone dependent tumor, uterine fibroid growth and development are related to ER and PR activity. Investigation into the mechanism of uterine fibroid genesis has shown the hormonemediated role of estrogen as the promoter of leiomyoma tumorigenesis [30, 31]. ER and PR immunohistochemical status has been reported to be important to affect the outcome of leiomyomas treated with radiofrequency ablation (RFA). The volume reduction rate and improvement in the symptom severity is significantly higher in the group having moderate to strong ER and PR expression than they were in the group with weak or negative ER and PR expression [32]. In this study, we didn't group our patients in the same way. Actually, we used patient as their own control by treating only a small part of uterus leomyoma, such that the treated area was an island in the whole lesion. We observed the difference of the status of ER and PR in both the targeted area and the normal tissue surrounding the targeted region. ER/ PR status was used as one of the indicators to identify the tissue necrosis. As a clinical application, identifying ER and PR expression status in HIFU-treated tumors may help predict tumor re-growth that lead to recurrent symptoms.

In current study, several layers of ER and PR positive cells in the transitional region were identified. In a prior study using RFA on uterine leiomyomas, ER and PR expression in the transition region were thought to be related to heat transmission from the electrode axis [22]. With focused ultrasound, a combination of thermal effects and cavitations primarily cause necrosis [19]. The temperature rise in the targeted tissues can be rapid and high, but some of this heat is carried away into surrounding tissue by thermal diffusion. If surrounding tissues are heated sufficiently, bioeffects can arise [33]. Positive expression of ER and PR in the transitional region may due to thermal diffusion or as a result of remaining viable tissue. Further investigation is needed to determine the etiology of this observation and significance of heat transmission beyond the HIFU focus.

Uterine leiomyomas are benign tumors containing a pseudocapsule between tumor and normal myometrium. From the previous studies in patients with uterine leiomyomas, larger treated tumor volume has been associated with improved symptomatic control [10, 12]. Current HIFU therapy for uterine fibroids is designed to treat the tumor while avoiding damage to the surrounding myometrium [34]. We particularly interested in the activity of ER/PR at the edge of HIFU therapy zone. The next hypothesis is that the vivid ER/PR around the HIFU treated region would be the source of recurrence of uterine fibroids after HIFU therapy. If this hypothesis will be verified in future, it could inform new strategies for HIFU treatment of uterine fibroids. For example, make the determination of how much tissue we should treat in different ER/PR status patients. However, the prognosis of uterus fibroids using HIFU therapy can not be addressed from these data.

Theoretically, broadening the HIFU treatment area to include the entire tumor would ablate all hormone-sensitive tumor tissue prevent tumor from recurring. Determining the presence of residual viable tumor using ER and PR expression may be helpful in this setting. In further studies, we will make strategies and explore the prognosis of HIFU treatment through long term follow up according to the classification of patients ER/PR status.

The main limitation of this paper is that this is an *ex vivo* study with the stationary treatment of uterine fibroids. Whether a margin of incompletely treated tissue would be present in the *in vivo* setting can not be addressed from this data. Further testing to examine ER and PR expression status in the transitional region after HIFU therapy from biopsy specimens may be helpful to confirm the utility of this technique. Another limitation of this study is the semi-quantitative approach used to determine the ER and PR expression in the transitional region is inaccurate. Anyway, it provided some basic information and a clue for further study.

Conclusion

In current study, the typical pathologic changes of coagulation necrosis in uterine fibroid tissue after treatment with ultrasound guided HIFU were verified by TTC, H&E staining, and immunohistochemical staining. The status of ER and PR could be a useful indicator of HIFU treatment effectiveness. The significance of the transition region demonstrated by ER and PR expression may help us in making therapeutic strategy and predicting prognosis according to patients' ER/PR status classification in future; however, it needs further study to determine.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xuemei He, College of Biomedical Engineering, Institute of Ultrasound Engineering in Medicine, Chongqing Key Laboratory of Biomedical Engineering, Chongqing Medical University, College Road, Yuzhong District, Chongqing 400016, China. Tel: +86 23 89012746; Fax: +86 2368811487; E-mail: hexm1978@163.com

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