Heat fixation of cancer cells ablated with high-intensity–focused ultrasound in patients with breast cancer

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Manuscript received July 29, 2005; revised manuscript March 22, 2006

Abstract

Background: High-intensity–focused ultrasound (HIFU) is a noninvasive thermal ablation technique. This study reports the use of histological techniques for the pathological assessment of HIFU effects in patients with breast cancer.

Methods: Twenty-three patients with biopsy-proven breast cancer underwent HIFU treatment for primary breast lesion. Mastectomy was performed on all patients after HIFU. By using histological examinations, the surgical specimens were assessed to explore HIFU effects on breast cancer.

Results: Coagulation necrosis of targeted tumors was confirmed by microscopy in 23 patients. Tumor cells presented typical characteristics of coagulation necrosis in the peripheral region of the ablated tumor in all patients. However, in 11 of 23 patients, hematoxylin and eosin staining showed normal cellular structure in the central ablated tumor. By using electronic microscopy and nicotinamide adenine dinucleotide-diaphorase stain, those who had normal-appearing cancer cells were not viable.

Conclusions: HIFU can cause the heat fixation of ablated tumor through thermal effect. © 2006 Excerpta Medica Inc. All rights reserved.

Keywords: High-intensity–focused ultrasound; Focused ultrasound surgery; Breast cancer; Thermal ablation; Ultrasound; Therapy; Breast; Neoplasm

High-intensity–focused ultrasound (HIFU) has recently been used as a noninvasive therapy to treat patients with solid malignancy [1–4]. No therapeutic instrument is inserted into a targeted lesion during the procedure; this makes HIFU potentially more attractive than other minimally invasive therapies for the local treatment of tumor, such as radiofrequency, laser, and microwave. In previous decades, animal studies showed that HIFU could induce coagulation necrosis in vivo of a targeted volume at depth, without damaging overlying tissue [5–10]. Three clinical trials designed for assessing the histopathologic changes have revealed obvious coagulation necrosis of solid malignancy treated with HIFU [11–13]. However, light microscopy alone may not always show coagulation necrosis of the treated tumor clearly [14,15], and the results may be limited by means of using conventional histological examination, such as hematoxylin and eosin (H&E) stain, for the assessment of the tumor destruction. Therefore, several histological examinations, including H&E, histochemical, and immunohistochemical stains, were performed in this study to evaluate therapeutic effects of HIFU on both cellular structures and viability of human breast cancer. The purpose of this study was to explore reliable histological methods for the assessment of HIFU effect on the tumor in patients with solid malignancy.

Methods

Patients

A total of 23 women with breast cancer were enrolled in this study, which was approved by the ethics committee at our university. At the time of enrollment, each patient signed an informed consent form, in accordance with the specification stipulated by the Helsinki Committee.
The selection criteria were as follows: histologically proven breast cancer (T1-2, N0-2, M0), single palpable mass, tumor size ≤ 6 cm, the lesion boundaries visualized with ultrasound imaging, and tumors separated by at least 0.5 cm from skin or rib cage and by more than 2 cm from the nipple. On these criteria, 27 patients were initially evaluated, of whom 4 (15%) were excluded in this study because of a lesion less than 2 cm from the nipple (n = 2), and the lesion margin was unclear on ultrasound imaging (n = 2).

The characteristics of 23 enrolled patients are summarized in Table 1. Breast cancer was confirmed by ultrasound-guided fine-needle aspiration in all patients before HIFU treatment. Each patient was treated with HIFU therapy before modified radical mastectomy, adjuvant chemotherapy, and radiation. They did not receive any intervention at the time of enrolment.

**HIFU device and treatment**

Mode-JC HIFU therapy system (Chongqing Haifu [HIFU] Tech Co, Ltd, Chongqing, China) is used in this study, and the technical details of this device have been previously described [16–21]. Briefly, this device is guided by real-time ultrasound imaging during the therapeutic procedure. The therapeutic ultrasound beam is produced by a 12-cm diameter piezoelectric ceramic transducer. It operates at a frequency of 1.6 MHz, and the focal length is 90 mm. The focal region is 3.3 mm along the beam axis and 1.1 mm in the transverse direction.

HIFU treatment was performed in all patients under either general anesthesia (n = 19) or intravenous sedation (n = 4). After suitable anesthesia, the patient was placed prone and immobilized on the treatment table so that the skin overlying the tumor was easily in contact with the degassed water. By using ultrasound imaging, a planning session was performed to identify the targeted tumor volume, which was subsequently divided into parallel slices of a 5-mm separation. To achieve a sufficient tumor-free margin, the HIFU-targeted volume included the breast lesion and marginal breast tissue 1.5 to 2.0 cm around the visible tumor.

Therapeutic parameters were delineated based on the position and depth of the target. Acoustic focal peak intensities for tissue exposure ranged from 5000 to 15,000 W/cm². By scanning the HIFU beam in successive sweeps from the deep to the shallow regions of the tumor, the targeted regions on each slice were completely ablated. The scanning spread of the therapeutic transducer ranged from 1 to 3 mm/s, and the track length was 20 mm. Median therapy time was 1.3 hours, ranging from 45 minutes to 2.5 hours.

**Histopathologic examinations**

By using standard surgical technique, modified radical mastectomy was performed in all patients 7 to 14 days after the HIFU treatment. Each breast specimen was kept at a low temperature and immediately submitted to pathology where it was examined grossly and histologically. Tissue blocks were sampled from the central and peripheral edges of the treated region and surrounding untreated normal breast tissue for assessing the effect of HIFU ablation on the breast cancer.

For electron microscopic examination, fresh tissue samples were put immediately into 2.5% glutaraldehyde, fixed at 4°C for 2 hours, and postfixed in 2% osmium tetroxide. Fixed samples were treated with a graded series of dehydration and then embedded in epon. Sections 0.1-μm thick were cut, stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope at 60 kV (Hitachi H-600, Tokyo, Japan).

The fresh samples were frozen, batched, and stored at −70°C for enzyme histochemical examination. Nicotinamide adenine dinucleotide-diaphorase (NADH) stain was performed on 8-μm cryostat-cut unfixed sections placed on glass slides for the analysis of tumor viability. Chemicals were obtained from Sigma-Aldrich Corporation (St Louis, MO). Each tissue section slide was incubated for 15 minutes at room temperature with 100 μL of incubation media, which consisted of 2.5 mg/mL of a-NADH 1 mL, 2 mg/mL of nitroblue tetrazolium chloride 2.5 mL, phosphate-buffered saline (pH 7.4) 1 mL, and Ringer solution 0.5 mL. Each slide was subsequently washed in distilled water for 2 minutes. Slides were assessed for characterization of staining during 24 hours of processing.

Finally, the remaining specimens were fixed in 10% phosphate-buffered formalin (pH = 7). They were embedded in paraffin, cut in 4-μm thick slices, and stained with H&E. By using the biotin-streptavidin-peroxidase immunohistochemical staining, the expression of carbohydrate antigen 15-3 (CA15-3) and vascular endothelial growth factor (VEGF) was determined in each removed breast cancer. The primary antibodies used were mouse monoclonal antihuman CA15-3 (Maxim Biotech Inc, San Francisco, CA) and antihuman VEGF (Santa Cruz Biotechnology, Santa Cruz, CA). The tumor cells were considered as positive when there was a homogeneous and clearly visible signal present in cytoplasm (brown) and negative if the signal was absent.
Evaluation and analysis

The gross and microscopic characteristics of the central and peripheral edges of the treated region and surrounding untreated normal breast tissue were recorded. Rates of complete and incomplete coagulation induced by HIFU ablation were descriptively documented. In immunohistochemistry staining, the positivities were assessed qualitatively.

Results

Histological examination

Immediately after HIFU treatment, local mammary edema was noted in all patients. Fourteen patients had mild local pain, warmth, and sensation of heaviness in the treated breast. Among them, 4 patients required a 3- to 5-day regimen of oral analgesics for pain relief. At the time of surgery, physical examination revealed that the local edema had almost disappeared, and a larger palpable lump was felt in all patients because the treated region consisted of the breast lesion and a 1.5- to 2.0-cm margin of adjacent normal breast tissue.

The ablated tumors were clearly defined in each patient. Coagulation necrosis of the targeted tissue, including the tumor and 1.5 to 2.0 cm of normal breast tissue surrounding the tumor, was confirmed by macroscopic observation in the 23 patients. It was gray-white or gray-yellow in color and felt firm on palpation. A red hemorrhagic ring was seen at the margin between the treated and untreated breast tissue; this might represent an inflammatory reaction of normal breast tissue to the ablation. However, there was no evidence of hemorrhage in the central region of the treated lesion.

Microscopic examination showed 2 aspects of histological change in the peripheral and central tissue of the ablated tumors, respectively. In the peripheral region, tumor cells had typical characteristics of coagulation necrosis induced by thermal energy, such as pyknotic nuclei, nuclear disruption, and disappearance, indicative of lethal and irreversible cell damage. Along the margin of the ablation, a narrow cellular band of fibrous tissue was identified, with the presence of fibroblasts, inflammatory cells, collagen fibrin, and capillary network (Fig. 1A). These changes were only observed at the peripheral region in all treated patients. However, the same tumor cell changes shown histologically in the peripheral region were only detected within the central region of the ablated tumor in 12 of 23 patients. In the remaining 11 patients, the central ablated tumors showed a unique set of additional features different from classical coagulation necrosis. H&E stain showed that cellular structure looked normal in some cancer cells, without any signs of tumor cell breakdown. The tissues maintained their cytologic staining characteristics and preserved nuclear chromatin, giving an appearance similar to viable cells (Fig. 1B).

Electron microscopy

Electron microscopy showed markedly varied cellular architectures of breast cancer cells between the peripheral region and the central region of treated tumor. In the peripheral region, the treated breast cancer had unrecognized amorphous electron-dense material (Fig. 2A). Plasma membrane, intracytoplasmic organelles, and nucleonic membrane were totally destroyed. The breast cancer architecture within the center of the treated tumor at low-power magnification was abnormal. Compared with breast cancer cells without any intervention (Fig. 2D), at high-power magnification the breast cancer cells revealed submicroscopic cellular damage that showed no morphologic coagulation necrosis by light microscopy. The tumor cells lacked nuclear membranes, and chromatin was clumped at the periphery of the nuclei. The cytoplasm contained some vacuoles, cell membranes were disintegrated, and organelle structures were not identified (Figs. 2B and C), suggesting an irreversible cell death occurred in these normal-appearing cancer cells.

Histochemical examination

NADH-diaphorase analysis for cell viability showed well-demarcated regions of negative staining within the treated region in all patients (Fig. 3C), consistent with complete loss of cellular viability. No pockets or foci of viable tumor cells were identified in the treated region compared with breast cancer cells untreated (Fig. 3D). There was no difference in this histochemical staining between the peripheral region and the central region of the ablated tumor, including the 11 samples that looked viable by H&E staining in the central region that showed nonviable cancer cells.
ability by NADH-diaphorase staining. However, the margin was very clear between the treated region (dead cells) and untreated region (alive cells), as shown in Figure 3A.

### Immunohistochemical staining

Both CA15-3 and VEGF positivities were confined to tumor cell cytoplasm. Twelve (52%) of 23 primary breast lesions treated with HIFU had a positive CA15-3 expression, and 7 (30%) of 23 expressed positive VEGF staining (Fig. 4). Among them, a CA15-3–positive expression occurred in 7 patients who had no morphologic necrosis by light microscopy and VEGF in 4 patients, respectively, as shown in Figure 4. Most of the positive tumors had multiple small foci of tumor cells exhibiting cytoplasm reactivity. Interestingly, the preserved and damaged cellular structure of breast cancer showed intense cytoplasmic positivity simultaneously, regardless of the histological changes under conventional light microscope.

### Comments

Ultrasound is a form of mechanical energy. It is generally believed that thermal effect and cavitation are directly involved in the tissue damage during HIFU exposure. The

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**Fig. 2.** Changes in cellular structure of tumor cells in patients with breast cancer after HIFU treatment. (A) At the peripheral region, breast cancer cells treated with HIFU present unrecognized amorphous electron-dense material, and it is difficult to identify plasma membrane and intracytoplasmic organelles (×6000). (B) At the central tumor, the cancer cells treated with HIFU contain some vacuoles in cytoplasm, cell membranes are disintegrated, and organelle structures are not clear (×3500). (C) The continuity of nuclear membrane is interrupted (arrow) in the HIFU-treated cancer cells (×12,000). (D) Subcellular structures in a removal sample of breast cancer without any intervention (a control) (×10,000).

**Fig. 3.** Histochemical changes in NADH activity at the marginal region of ablated tumor after HIFU treatment. (A) The treated tissue (a) presents negative staining, and the untreated tissue (b) shows positive staining (blue color). A clear margin (arrows) is detected between the treated and untreated regions (NADH-diaphorase histochemical stain ×400). (B) H&E-stained adjacent section of the same sample treated with NADH-diaphorase staining. The treated tissue (a) presents typical characteristics of coagulation necrosis in the peripheral part of ablated tumor, and granulation tissue (b) is observed along the margin of the ablation (arrows) (H&E stain ×100). (C) The negative staining of nonviable breast cancer treated with HIFU (NADH-diaphorase histochemical stain ×400). (D) Positive expression of NADH-diaphorase staining in a removal sample of breast cancer without any intervention (a control) (NADH-diaphorase histochemical stain ×400).

**Fig. 4.** Immunohistochemical changes in the expression of tumor associated antigens VEGF and CA15-3 after HIFU treatment (SP immunohistochemical stain ×400). (A and B) CA15-3–positive expression (brown color) in the treated cancer cells (arrow) that have nuclear disruption and disappearance (A), or thermally ablated tumor cells with an appearance similar to viable cells (B). (C and D) Positive expression (brown color) of VEGF in the treated cancer cells that have pyknotic nuclei (C) or thermally ablated tumor cells with an appearance similar to viable cells (D).
assessment of the HIFU efficiency in tumor ablation requires pathological confirmation. Previous animal studies revealed that H&E stain, a routine histological method, was a standard method to verify the viability of the treated cells after HIFU ablation [5–10]. By using this staining, clear evidence of complete coagulation necrosis could be clearly identified in the treated tissue under light microscope. In this clinical study, H&E staining showed complete coagulation necrosis of the treated tumor in 12 patients (52%) with breast cancer, the same histological results as found in the animal studies. But in the remaining 11 patients (48%), the cellular structures of breast cancer were preserved in the central part of the ablated tumor, and the tissues also maintained their cytological and nuclear staining characteristics. Similar findings have been previously reported in a patient with metastatic liver cancer after HIFU treatment [22]. This raised the question of whether incomplete destruction had occurred in the ablated tumors. However, electron microscopy revealed that those who had normal-appearing cancer cells, with light microscopy, showed an irreversible cell death, indicating the preservation of cellular structure induced by thermal fixation, instead of incomplete coagulation necrosis. The high temperature, which was caused by HIFU exposures in the treated region, appeared to result in either cell death or the denaturation of enzyme protein constituents, similar to the preservation seen with formalin fixation. As a result of thermal fixation, the central part of the ablated tumor resisted degradation because the wound-healing process could not extend this region immediately after HIFU treatment. In contrast, in the peripheral region, the local response resulted in the formation of granulation tissue, neutrophil and monocyte infiltration, and activated fibroblasts that can breakdown, resolve, and repair sites of coagulation necrosis.

Furthermore, demonstration of cytological enzyme activity was used in this study to develop an alternative method for identifying cell viability more easily. This enzyme histochemical analysis is based on the reduction of nitroblue tetrazolium chloride, a redox indicator, by NADH-diaphorase, resulting in an intense blue cytoplasmic pigment. The activity of this enzyme has been shown to subside immediately on cell death [23,24]. In this study, we found that H&E staining of cell necrosis was associated directly with cell nonviability by NADH-diaphorase. Also, within the ablated tissue, areas that seemed to be normal by H&E staining were deemed nonviable by NADH-diaphorase staining. This suggests that NADH-diaphorase stain is more accurate and more objective than H&E staining in assessing acute cell death because it is based on the presence or absence of enzyme function instead of changes in cellular structure. Therefore, the combination of H&E and NADH-diaphorase stains should be performed to confirm whether the treated tumor is viable or nonviable in the tissue samples obtained from core biopsy after HIFU ablation. Follow-up biopsy provides scanty tissue making interpretation as to whether there has been complete ablation of the tumor very difficult. As such, follow-up radiologic examinations, such as contrast ultrasound, enhanced computed tomography scan, or magnetic resonance imaging, provide alternative modalities to assess the whole ablative appearance of targeted tumors after HIFU ablation. They can show the changes in the vascular perfusion of either tumor or normal breast tissue and distinguish the difference between nonviable and residual viable tumor directly in the treated regions. CA15-3 is a high–molecular-weight (300–450 kDa) polymorphic epithelial mucin. It has a high expression in breast cancer cells, which is usually related to clinical stage and progression [25,26]. VEGF is a heparin-binding molecule (46 kDa), which plays an active role as an endothelial cell-specific mitogen and as a vascular permeability factor. Expression of VEGF in breast cancer has been shown to increase tumor growth, angiogenesis, and metastases [27,28]. Although high temperatures could induce protein denaturation during HIFU exposure, it was unclear whether this change would have an effect on the expression of associated tumor antigens such as CA15-3 and VEGF in the denaturated breast cancer after HIFU treatment. Therefore, immunohistochemical staining was performed in this study to detect subtle phenotypic changes in the breast cancer and to explore the feasibility of using cellular markers for the necrosis induced by HIFU. However, CA15-3–positive expression was confirmed in 52% patients receiving HIFU treatment and VEGF in 30% patients, respectively, despite the histological changes. As a result, this method is not sufficient to confirm the coagulation necrosis caused by HIFU ablation.

There are several problems encountered in HIFU ablation for breast cancer. General anesthesia may be problematic in an HIFU procedure. It can be explained by the facts that general anesthesia could be necessary at the early development of a new technique. However, other studies indicated that either local anesthesia or intravenous sedation was feasible during the procedure for the thermal ablation of breast cancer [12,29], and this result may change our selection of anesthetic methods in the future. Patients whose tumor is close to the skin are not suitable candidates for HIFU treatment because there is a possibility of causing skin burn or leaving residual cancer cells.

The lack of reliable histological techniques for assessing coagulation necrosis induced by HIFU may complicate the application of this noninvasive treatment for patients with malignancy. This study reveals that pathological changes in HIFU-ablated tumor are comprised of not only obvious tissue destruction detected by light microscopy but also thermal fixation of the tumor. The thermally fixed tissue is usually located in the center of the tumor, and cytological and nuclear staining characteristics are normally maintained with a similarity to viable cells. When using H&E staining, it is difficult to identify fixed cancer cells from dead cells. However, histochemical demonstration of cytological enzymatic activity can be used as an alternative method for distinguishing morphologic difference between classical ne-
crosis and fixed tissue. Thus, it is concluded that HIFU can cause the heat fixation of ablated tumor through thermal effect, and the combination of both H&E and NADH histochemical stains could provide a reliable assessment for histological analysis of therapeutic effects on tumor cells after HIFU treatment.

References