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Histopathological effect of radiofrequency ablation therapy for primary breast cancer, with special reference to changes in cancer cells and stromal structure and a comparison with enzyme histochemistry

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Abstract Radiofrequency ablation (RFA) therapy is expected to be applicable to small breast cancers, but no criteria for its histopathological effect have yet been established. Using samples obtained from 15 patients who had undergone RFA and subsequent mastectomy, we compared the histopathological changes in the ablated area with the results of histochemical staining based on the reduction of nitroblue tetrazolium chloride (NBT) by nicotinamide adenine dinucleotide (NADH) diaphorase in frozen tissue sections, and looked for histological changes indicative of the effect of RFA on breast cancer. Grossly, the ablated area in most of the tumors was rough, gritty, less moist, and surrounded by a red congestive limbic zone. The ablated area showed no staining by the NADH diaphorase reaction, and cancer cells in the area showed marked destruction characterized by an unclear intercellular boundary, elongated eosinophilic cytoplasm, pyknotic "streaming" nuclei, and a poorly defined nuclear and cytoplasmic texture. At the same time, fibrous connective tissue also showed degenerative changes, becoming densely homogeneous with loss of its delicate wavy structure. The area in which RFA appeared to have been histopathologically effective was mostly concordant with the area in which the NADH diaphorase reaction was negative. In the periphery of the ablated area, however, cellular changes caused by RFA were less marked, although the NADH diaphorase reaction was visualized with NBT. A larger number of cases should be examined in order to establish criteria for the histopathological effect of RFA on breast cancer.

Keywords Breast cancer · Radiofrequency ablation therapy · NADH diaphorase reaction · Histopathological criteria for therapeutic effect

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Introduction

Radiofrequency ablation (RFA) therapy is expected to be applicable to small breast cancers as an effective and safe curative treatment of choice. However, no criteria for defining its therapeutic effect have yet been established. The majority of previous studies have employed histopathological examination of hematoxylin–eosin (HE)-stained sections and the histochemical technique for visualizing the reduction of nitroblue tetrazolium chloride (NBT) by nicotinamide adenine dinucleotide (NADH) diaphorase in frozen sections.

The NAD+/NADH redox reaction is one of the most important in living biologic systems. NADH diaphorase activity judged from the reduction of NBT to formazan via oxidation of NADH is a reliable marker of cell viability. Assay of NADH diaphorase is performed histochemically using fresh frozen tissue sections. When reduced NADH is

oxidized by NADH diaphorase, free electrons are transferred to NBT, which becomes reduced and converted to the blue, water-insoluble dye formazan (Fig. 1). NADH diaphorase becomes bound to the structural components of the cell, thereby permitting histochemical visualization of its intracellular location by the use of NBT. Only viable cells have active diaphorase, whereas this activity seems to subside immediately after cell death.

On the other hand, several previous reports have described criteria for evaluation of the RFA effect. Jeffrey et al. [1] considered the presence of pyknotic nuclei and increased intensity of eosinophilic staining to be characteristics of tissue cautery due to heating. Earashi et al. [2] applied histopathological criteria for assessment of therapeutic response described in the "General Rules for Clinical and Pathological Recording of Breast Cancer". However, no histopathological criteria for the therapeutic effect of RFA have yet been established.

In the present study, on the basis of a comparison of histopathological changes in the ablated area with the results of histochemical assay with NADH diaphorase, we attempted to characterize the histological changes in breast cancer induced by RFA.

Patients and methods

RFA study protocol

Patient selection and the RFA protocol have been described previously [3]. Histochemical and histopathological examinations were performed on specimens from 15 patients who had undergone RFA for primary breast cancer and subsequent mastectomy between June 2006 and May 2007.

Pathological analysis

After ablation, the surgically resected specimen was cut at the maximum diameter of the ablated breast tumor (Fig. 2). Both the ablated and non-ablated areas of each tumor and adjacent tissue were grossly evaluated, focusing particularly on the features of coagulation, congestion, and elasticity. Slices of tissue, each including apparently ablated and non-ablated areas, were obtained and mounted in optimal cutting temperature (OCT) compound. The tissue was then immediately frozen in liquid nitrogen, and cut into sections 8- to 10-μm thick. One of these sections was immediately stained with HE, and the others were stored at −80°C until NADH diaphorase-NBT studies. The remaining surgically resected specimens were fixed in 10% formalin and processed for routine histopathological examination.

Enzyme histochemical analysis

For enzyme histopathological analysis of ablated breast tumors, frozen tissue sections were incubated for 1 h at 37°C in a solution consisting of 0.8 mg/mL reduced β-NADH (Sigma), 0.5 mg/mL nitroblue tetrazolium (Sigma), and 0.05 M Tris-buffered saline (pH 7.4) (Fig. 3). Each slide was fixed in 10% formalin for 30 min and washed in distilled water for 2 min, then glass coverslips were applied with an aqueous medium.

Mapping and evaluation

Ablated cells were confirmed to be non-viable by their negativity for the oxidation–reduction reaction mediated by NADH diaphorase, whereas residual viable cells were stained blue. By referring to serial sections stained with

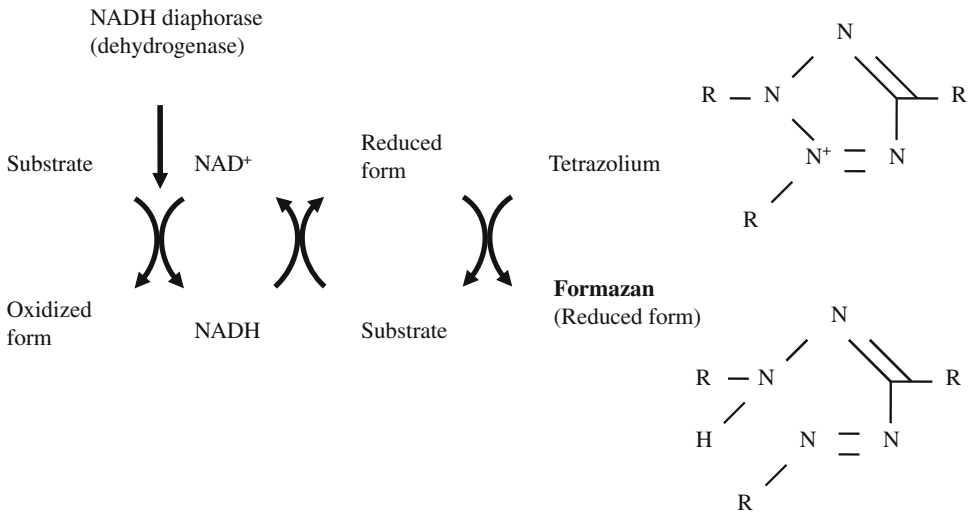

Fig. 1 Nicotinamide adenine dinucleotide (NADH) redox circuit

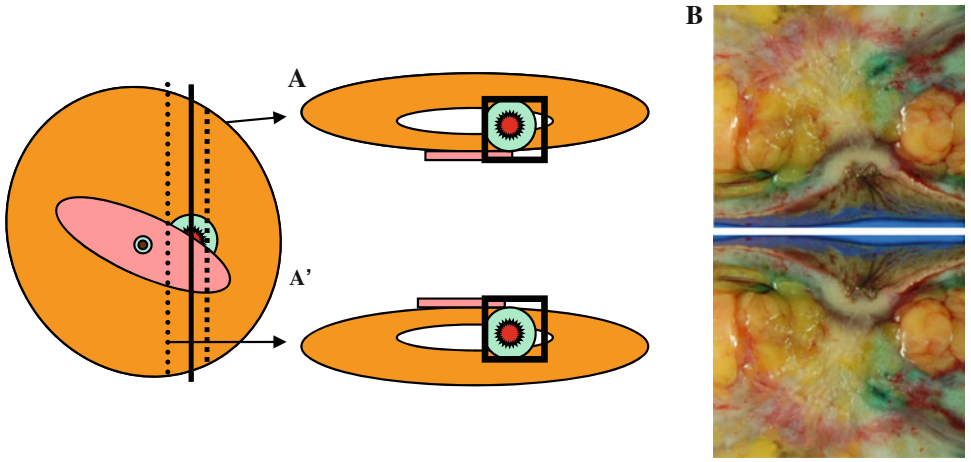

Fig. 2 Schematic representation of tissue specimens used for evaluation of the histopathological effect of radiofrequency ablation (RFA) to primary breast cancer. **a** An ablated tumor cut at the maximum diameter. The tumor in cut section **a** is taken for NADH diaphorase staining. The tumor in cut section **a′**, the mirror image of section **a**, is taken for routine formalin-fixed and paraffin-embedded blocks. **b** Gross features of the ablated tumor. A congestive limbic zone encircles the ablated area containing the tumor

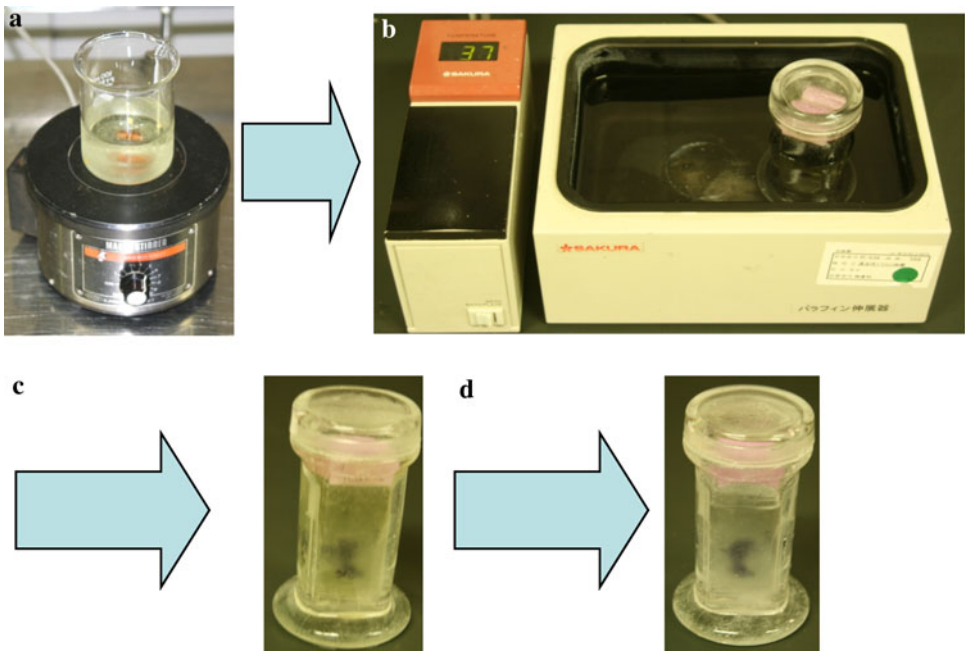

Fig. 3 Preparation of reagents for histochemical assay of nicotinamide adenine dinucleotide (NADH) diaphorase activity. **a** Adjustment of NADH medium. The incubation medium consists of 0.8 mg/mL reduced β-NADH (Sigma), 0.5 mg/mL nitroblue tetrazolium, and 0.05 M Tris-buffered saline (pH 7.4), mixed at 37°C. **b** Fresh frozen tissue sections are incubated in the NADH medium in a water bath at 37°C for 1 h. **c** The tissue sections are washed in distilled water for 2 min. **d** These sections are subsequently fixed in 10% formalin for 30 min

both the NADH diaphorase reaction and HE, we compared the histopathological features of stained and adjacent non-stained areas. Gross and histological features attributable to the thermal effects of RFA were investigated by two pathologists (K.S. and H.T.).

Results

Gross examination

At the cut surface including the tumor, the ablated area felt firmer and more fragile than the surrounding non-ablated area. The cut surface of the ablated area composed of tumor and fibrous stroma was rough, gritty, and less moist, forming a round, flat surface surrounded by swollen fresh, unablated mammary, and fibroadipose tissue (Fig. 4a). In the central zone of the ablated area, the tumor and fibrous connective tissue were grayish-white to tan in color, forming a fissure or small cavity around the needle track (Fig. 4a). Coagulated non-tumor fibroadipose tissue was also firm and had changed to a tan-yellowish color. A red congestive limbic zone surrounded the ablated area (indicated with dots in Fig. 4a). These congestive rings were observed in 14 of 15 cases.

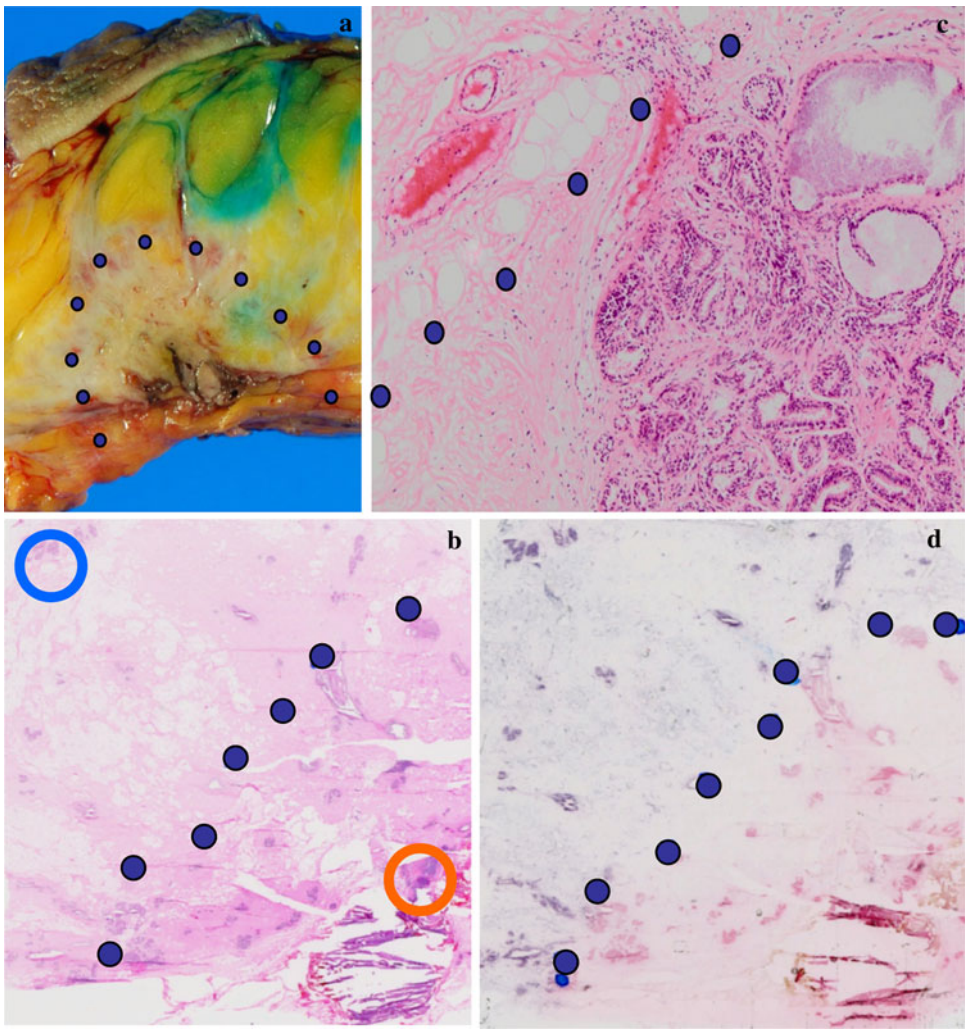

Fig. 4 Macro- and microscopic features of RFA-treated breast cancer and non-cancerous tissue. **a** Gross features of a mastectomized specimen resected immediately after the RFA procedure. The border between the ablated and non-ablated areas is delineated by a congestive limbic zone (indicated by *dots*). **b** The boundary between the ablated area (*right lower* to *dots*) and non-ablated area (*left upper* to *dots*). A low-magnification view of an HE-stained section. The control part of the ablated area (*red circle*) shows highly degenerative changes. **c** A higher-magnification view of the boundary between the ablated (*right lower* to *dots*) and non-ablated (*left upper* to *dots*) areas. In the latter, congestive blood vessels are evident. In the former, mammary tissue and stroma with mild to moderate heating effects are observed (HE). **d** The boundary between the ablated and non-ablated areas in the serial section of image **b**. The section was subjected to the NADH diaphorase reaction to color viable cells blue due to the reduction of NBT. Only the non-ablated area (*left upper* to *dots*) is stained blue

Microscopy examination

The boundary between the ablated and non-ablated area was identifiable histologically, although the effect of cautery showed a gradation from strong in the center to mild or moderate at the periphery (Fig. 4b, c). RFA damage to the epithelial cells and fibrous stroma in the ablated area was histologically visualized as follows in HE-stained sections. Epithelial cells, both cancerous and non-cancerous, were characterized by elongated eosinophilic cytoplasm with pyknotic "streaming" nuclei (Fig. 5b). The intercellular boundary and details of the nuclear and cytoplasmic texture were unclear. Fibrous connective tissue also showed degenerative changes resulting in dense homogeneous and highly eosinophilic features (Fig. 6). The original delicate, wavy structure had entirely disappeared. Fibroblasts in the area also showed thermal degenerative changes identical to those seen in epithelial cells.

At the periphery of the ablated area, epithelial cells showed coarse and plain nuclear chromatin due to the thermal effect (Fig. 5c). The boundary between ablated and non-ablated areas was usually characterized by congestive blood vessels, which were grossly evident as a red limbic zone (Fig. 4a). The effects of heating were sometimes relatively mild to moderate near the limbic zone, because the nuclear and cytoplasmic features characteristic of cell death at the periphery of the ablated area adjacent to the red zone were less marked (Fig. 5c) than those in the central ablated area.

Comparison between NADH diaphorase reaction and histopathological findings

Nicotinamide adenine dinucleotide diaphorase-stained sections showed no reaction in tumor cells at the center of the ablated area, where tumor cells and stroma showed marked heat degeneration. The border between the NADH diaphorase-positive and -negative areas was relatively clear and sharp (Fig. 4d). NADH-positive cells showed the fine structures of intact nuclear chromatin and cytoplasm

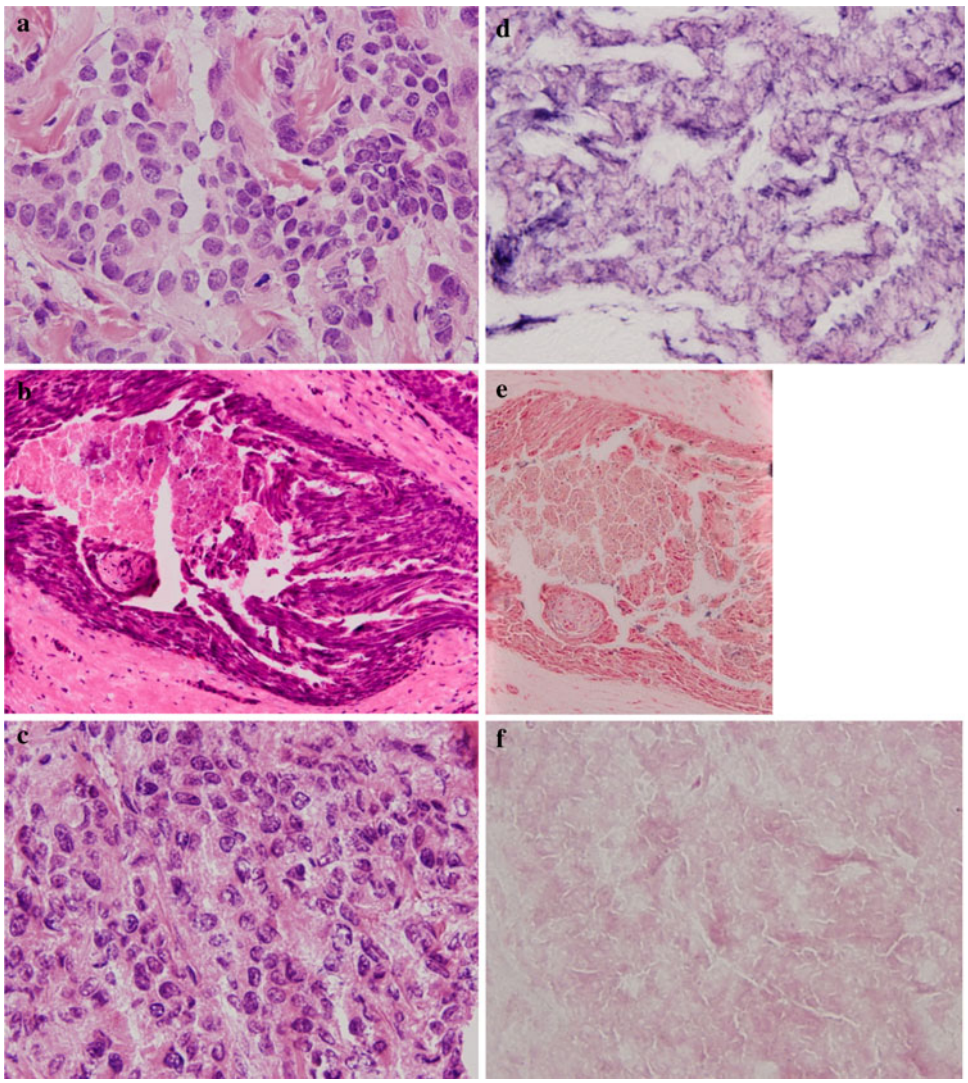

Fig. 5 Comparison of histopathological features of RFA-treated breast cancer tissue with histochemical results of NADH diaphorase staining. **a**, **d** A non-ablated invasive ductal carcinoma (control specimen). **b**, **e** An invasive ductal carcinoma showing a strong effect of RFA cautery. **c**, **f** An invasive ductal carcinoma showing a moderate heating effect of RFA. **a** Fine structure of the nuclei and cytoplasm of tumor cells, and the collagen fibers of the stoma, are preserved. **b** Ablated tumor cells show an elongated cytoplasm with "streaming-like" nuclei. **c** Ablated tumor cells are characterized by pale cytoplasm and rough chromatin in the nuclei with an unclear cellular border. **d** This carcinoma shows a positive NBT reduction reaction, staining the cells blue, indicating histochemical positivity for NADH diaphorase activity. **e** This carcinoma shows a negative reaction for NADH diaphorase, indicating an absence of viable tumor cells. An invasive ductal carcinoma showing a strong cautery effect of RFA. **f** This carcinoma shows a negative reaction for NADH diaphorase, indicating an absence of viable tumor cells

(Fig. 5a, b), surrounded by a fine, delicate fibrous stroma. In each tumor, the NADH-negative area was approximately equivalent to the area circumscribed by the congestive limbic zone. Neither the central area showing strong effects of cautery, nor the peripheral part of the ablated area showing less marked effects showed the NADH diaphorase reaction (Fig. 5d, f).

Discussion

We have described the macro- and microscopic findings characteristic of the cautery or heating effect of RFA, based on examination of specimens resected immediately after the procedure. The histopathological features of cellular damage described by some authors have included an unclear intercellular boundary, elongated eosinophilic cytoplasm, pyknotic "streaming" nuclei, and poorly defined nuclear and cytoplasmic texture [1, 4]. In addition, we found that the RFA procedure caused fibrous connective tissue to lose its delicate wavy structure and to degenerate to dense eosinophilic tissue with a loss of fine structure.

The area in which RFA was histologically effective was mostly concordant with the area of NADH diaphorase negativity, especially in the central part of the ablated area. At the periphery, however, cellular change caused by RFA was less marked, and the NADH diaphorase reaction visualized by NBT was usually negative. Fornage et al. described the histopathological changes observed in RFA-treated breast tissues in a study of 21 patients. The changes were similar to those observed in the present study, and were concordant with the results of NADH diaphorase staining [4]. However, a large number of cases should be studied in order to establish criteria for the histopathological effect of RFA. Although the histochemical assay for NADH diaphorase activity is reliable, it cannot always be performed in routine practice. It will therefore be necessary

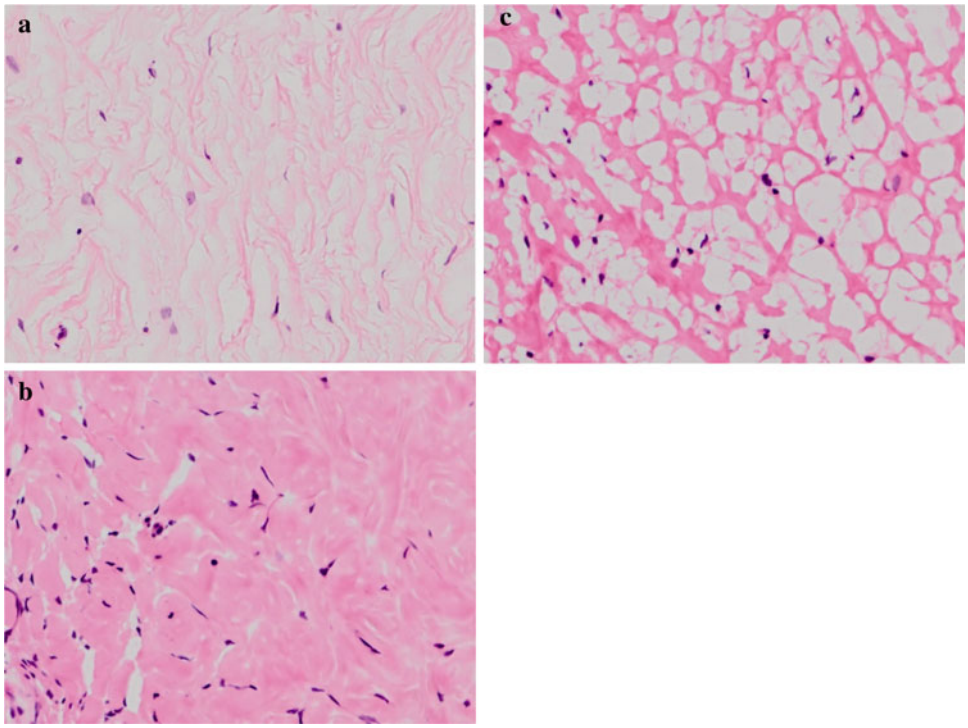

Fig. 6 **a** Non-ablated stromal tissue in the breast demonstrates the fine wavy structure of collagen fibers (control specimen). **b**, **c** A highly ablated stroma showing eosinophilic and amorphous features without a fine wavy texture. Nuclei of fibroblasts are also pyknotic

to standardize criteria for the effects of RFA that are applicable to formalin-fixed and paraffin-embedded tissue sections.

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