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ORIGINAL ARTICLE

Label-free detection of residual breast cancer after neoadjuvant chemotherapy using biomedical multiphoton microscopy

Zhonghua Han[1] · Lianhuang Li[2] · Deyong Kang[3] · Zhenlin Zhan[2] · Haohua Tu[4] · Chuan Wang[1] · Jianxin Chen[2]

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Abstract

Neoadjuvant chemotherapy has become a standard treatment for breast cancer as it has been shown to increase the rate of breast preservation and to improve outcome in patients. However, how to accurately detect residual tumors is still a challenge. In this work, we tried to use multiphoton imaging to look for residual breast tumors after preoperative therapy. Imaging results demonstrate that multiphoton microscopy can identify remaining tumor tissues and can even detect rarely residual tumor cells, which would be helpful for surgeons to accurately assess the surgical margin in real time to confirm negative margins during operation. We also performed a quantification analysis of the nuclear area of tumor cells before and after treatment with neoadjuvant chemotherapy. The measurement data show that the tumor cell nuclei after chemotherapy are significantly larger than those without treatment, and there is a statistically significant difference in the nuclear areas between the pre-treatment and post-treatment mammary carcinoma. Our pilot study indicates the potential utility of multiphoton imaging for detecting residual breast carcinoma tissues in fresh, ex vivo specimens without the use of exogenous contrast agents. We foresee real-time intraoperative applications of multiphoton microscopy in evaluating therapy response, and thereby helping clinicians develop individualized treatment plans.

Keywords Neoadjuvant chemotherapy · Breast cancer · Multiphoton microscopy

Introduction

Breast cancer is a common malignancy in women all over the world and has a high mortality rate. Surgery has long been a treatment method, but it has not worked as well as people wish. Neoadjuvant chemotherapy for breast cancer was originally used in locally advanced inoperable disease in order to achieve surgical resection [1, 2]. At present, preoperative chemotherapy is frequently utilized in the management of mammary carcinomas because it has been shown to reduce tumor size and to improve outcome substantially [3, 4]. By downstaging the tumor, less extensive resections are needed, and this often makes breast preservation feasible for patients who would otherwise have a mastectomy [5, 6]. However, how to accurately monitor tumor response is a serious problem, especially to detect scarcely residual tumor cells.

Although mammography is the primary clinical imaging modality used to examine breast cancer, it involves radiation, and therefore, frequently repeating mammograms during primary chemotherapy for monitoring responses is not acceptable [7–9]. Ultrasonic examination is a very useful imaging technique and is widely used to evaluate the efficacy of neoadjuvant treatment; however, it is unable to distinguish tumors from fibrosis [10]. More importantly, limitations in resolution of these two imaging techniques have led to the development of alternative techniques. Magnetic resonance imaging (MRI) has a growing role in the management of breast cancers [11, 12], but its resolution cannot reach the level of a single cell

✉ Lianhuang Li
lhli@fjnu.edu.cn

[1] Department of Breast Surgery, Fujian Medical University Union Hospital, Fuzhou 350001, People's Republic of China

[2] Key Laboratory of OptoElectronic Science and Technology for Medicine of Ministry of Education, Fujian Provincial Key Laboratory for Photonics Technology, Fujian Normal University, Fuzhou 350007, People's Republic of China

[3] Department of Pathology, Fujian Medical University Union Hospital, Fuzhou 350001, People's Republic of China

[4] Beckman Institute for Advanced Science and Technology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

too. Consequently, new detection technologies are needed that can overcome the limitations of resolution.

Multiphoton imaging is an advanced imaging technology and offers many advantages over confocal microscopy including low photo-damage and light-bleaching, high penetration depth, as well as 3D imaging thanks to optical sectioning, and thereby has been widely used in life science research [13–15]. Two-photon excited fluorescence (TPEF) and second-harmonic generation (SHG) are the most widespread multiphoton phenomenon involved in multiphoton microscopy (MPM). Compared with other conventional imaging techniques, the most important advantage of this technique is the ability to provide subcellular-resolution images from unprocessed and unstained tissues [16], where TPEF imaging can instantly visualize cellular details and SHG imaging may provide additional information on the extracellular matrix. Until now, although this imaging technique still cannot realize in vivo whole body imaging, it has been used extensively to study multiple organs including the colorectum, stomach, and lung and skin cancers [17–20]. Therefore, this work was undertaken to evaluate the ability of multiphoton microscopy combined TPEF with SHG imaging to accurately show residual breast malignancy in women treated with neoadjuvant chemotherapy.

Materials and methods

Sample preparation

This study was conducted with the approval of the Institutional Review Board at the Fujian Medical University Union Hospital, and written informed consent was obtained from all individual participants included in the study. In this work, 15 patients with breast carcinoma were recruited and their information was summarized in Table 1. Once diagnosed, all patients received a standard neoadjuvant chemotherapy protocol consisting of 4 courses of EC (100 mg/m^2 epirubicin and 600 mg/m^2 cyclophosphamide) every 3 weeks, followed by 4 courses of docetaxel 75 mg/m^2 every 3 weeks, or 12 courses of paclitaxel 80 mg/m^2 administered weekly. Moreover, HER2-positive breast cancer was administered additional trastuzumab during the docetaxel or paclitaxel treatment every 3 weeks.

A total of 30 fresh breast cancer tissues including 15 pre-treatment and 15 post-treatment cancer tissues were collected. Each sample was serially sectioned at 10 μm thickness by cryostat microtome in the pathology laboratory, and one section was used for multiphoton imaging, while an adjacent section was stained with hematoxylin and eosin (H&E) for pathological examination. A small amount of phosphate-buffered saline (PBS) was applied to the specimen to avoid dehydration or shrinkage during the imaging process. All the H&E-stained slices were firstly reviewed by a certified pathologist, and then the H&E-stained images were taken using a standard bright field light microscope (Eclipse Ci-L, Nikon Instruments Inc., Japan) with a CCD (DS-Fi2, Nikon). Lastly, the imaging results from multiphoton microscopy were compared with the H&E-stained images for confirmation.

Multiphoton imaging system

The imaging system is mainly based on a commercially upright laser scanning microscope (LSM 880, Zeiss, Germany) combined with a mode-locked femtosecond Ti:sapphire laser (Chameleon Ultra, Coherent, Inc., USA). An excitation wavelength of 810 nm was used for multiphoton imaging including TPEF imaging and SHG imaging, and an oil immersion lens (Plan-Apochromat × 63, NA = 1.4) was used for focusing the excitation beam into samples and for obtaining high-resolution imaging. The TPEF signal was collected from 430 to 716 nm by a 32-channel GaAsP PMT array detector, while the SHG signal was collected from 389 to 419 nm by a GaAsP PMT. The images were obtained at 1.8 μs per pixel, and the scanning speed was 2 frames/second (512 × 512 pixels) in our imaging system. An array of images with a resolution of 512 × 512 pixels were acquired and assembled to form a large-area image. All the images had a 12-bit pixel depth. Additionally, TPEF image was color coded in red and SHG image was color coded in green to increase the contrast.

Statistical analysis

To determine whether there were changes in tumor cells between the pre-treatment and post-treatment breast cancer tissues, the nuclear area was measured from MPM images by the ZEN imaging software in Zeiss LSM880 System. These final results were expressed as mean value and standard deviation (SD). The student's t test was used for assessing the statistical significance, and $P \leq 0.05$ was regarded as statistically significant. All statistical analyses were performed using the IBM SPSS Statistics 21.

Results

Using MPM to image normal breast tissues

The aim of this study was to assess the potential of multiphoton microscopy as a diagnostic tool to identify residual breast tumors in fresh tissues that was unprocessed and unstained. To do this, the morphology of normal tissue was first revealed by MPM imaging for comparison. Figure 1 displays representative multiphoton images of normal breast tissue and corresponding H&E-stained image. As you know, normal mammary gland mainly consists of breast ducts, lobules, and

Table 1 Patient demographics

Patient no.	Age	Pre-treatment tumor stage	Pre-treatment tumor size (cm × cm)	Post-treatment tumor stage	Post-treatment tumor size (cm × cm)
1	34	cT2N2M0	4.0 × 3.5	ypT1N2M0	2.8 × 1.6
2	54	cT3N3M0	5.0 × 6.0	ypT2N3M0	4.0 × 3.5
3	49	cT2N1M0	4.4 × 3.5	ypT1N1M0	2.3 × 2.0
4	53	cT2N0M0	4.6 × 3.2	ypT0N0M0	0
5	39	cT3N3M0	5.5 × 3.3	ypT2N3M0	4.1 × 3.2
6	73	cT2N1M0	4.3 × 2.4	ypT3N0M0	2.5 × 2.0
7	41	cT3N2M0	6.2 × 4.8	ypT3N1M0	5.1 × 4.2
8	54	cT2N2M0	4.7 × 2.6	ypT1N2M0	3.0 × 2.0
9	39	cT2N2M0	6.0 × 4.8	ypT2N0M0	3.0 × 3.2
10	53	cT2N2M0	5.4 × 3.6	ypT2N1M0	4.0 × 3.5
11	43	cT3N2M0	5.0 × 3.4	ypT2N1M0	2.3 × 2.0
12	49	cT3N1M0	6.0 × 5.0	ypT2N0M0	4.0 × 3.2
13	42	cT3N2M0	8.6 × 7.9	ypT3N2M0	6.0 × 5.0
14	53	cT2N1M0	3.8 × 4.7	ypT1N1M0	2.4 × 2.0
15	44	cT3N3M0	8.0 × 7.0	ypT3N3M0	6.8 × 3.4

connective tissues. SHG image shows abundant collagen fibers in the extracellular matrix, while TPEF image clearly presents the tissue structure of ducts and lobules. More narrowly, the mammary duct is composed of many epithelial cells and is surrounded by collagen fibers that can be seen more clearly in the magnification multiphoton image (Fig. 1e). The terminal duct-lobular units are mainly made up of acini, and collagen fibers also encompass the acinus, which is more clearly shown in the magnified multiphoton image (Fig. 1f) too. These microstructural features obtained from MPM imaging agree well with the corresponding H&E-stained image of paired histological section (Fig. 1d).

Using MPM to identify breast tumor tissues

Researches were next undertaken to image tissue sections obtained from the breast mass before treatment. Figure 2 displays representative multiphoton images of invasive ductal carcinoma of the breast and corresponding H&E-stained images. It can be clearly seen that when tumors invaded, normal tissue structure would be destroyed and replaced. Concretely, SHG image shows that a great many of collagen fibers in stroma have disappeared and only a few fragmented collagen fibers (pink arrow in Fig. 2a) can be found. This information cannot be directly obtained through the H&E-stained image. On the other hand, TPEF image presents that normal breast tissue has been completely damaged and was replaced by a large number of tumor cells. As shown in Fig. 2e, the breast tumor cells can be exhibited more clearly by the zoom-in image of the boxed region in Fig. 2c. These morphologic features are well in agreement with those identified with the corresponding H&E-stained image (Fig. 2d).

Using MPM to detect residual breast tumors after neoadjuvant treatment

Then, study was implemented to image tissue sections obtained from the breast tumors after preoperative therapy. Figure 3 presents representative multiphoton images of invasive ductal carcinoma after neoadjuvant chemotherapy and corresponding H&E-stained image. It can be seen that tumor regression is obvious, and a severe fibrotic reaction could be observed compared to the pre-treatment breast tumor tissues. More specifically, lots of collagen fibers appear by SHG image because of tissue repair by fibrosis, and some are in chaos (Fig. 3f), while some are regular and have multilayer architecture (Fig. 3e). These features are more excellent than what is obtained using standard H&E histology. Some elastic fibers (yellow arrow in Fig. 3b) which are normally long rope-like were also found via TPEF signal. They have been destroyed, became fragmented and aggregated together caused by previous tumor infiltration. More importantly, residual breast tumor cells could be detected too. These tumor cells have various patterns: some may show as a single cell (blue arrow in Fig. 3e), while some may present as a nested architecture surrounded by fibrous stroma (white arrow in Fig. 3f). These morphological alterations correlate with the corresponding H&E-stained image of paired histological section (Fig. 3d). These experimental results demonstrate that multiphoton imaging can not only directly detect tumor cells left after preoperative chemotherapy, but also monitor the stromal changes caused by the pre-treatment.

Quantitative analysis

Furthermore, to determine whether there were changes in tumor cells prior to and after therapy, nuclear areas were measured

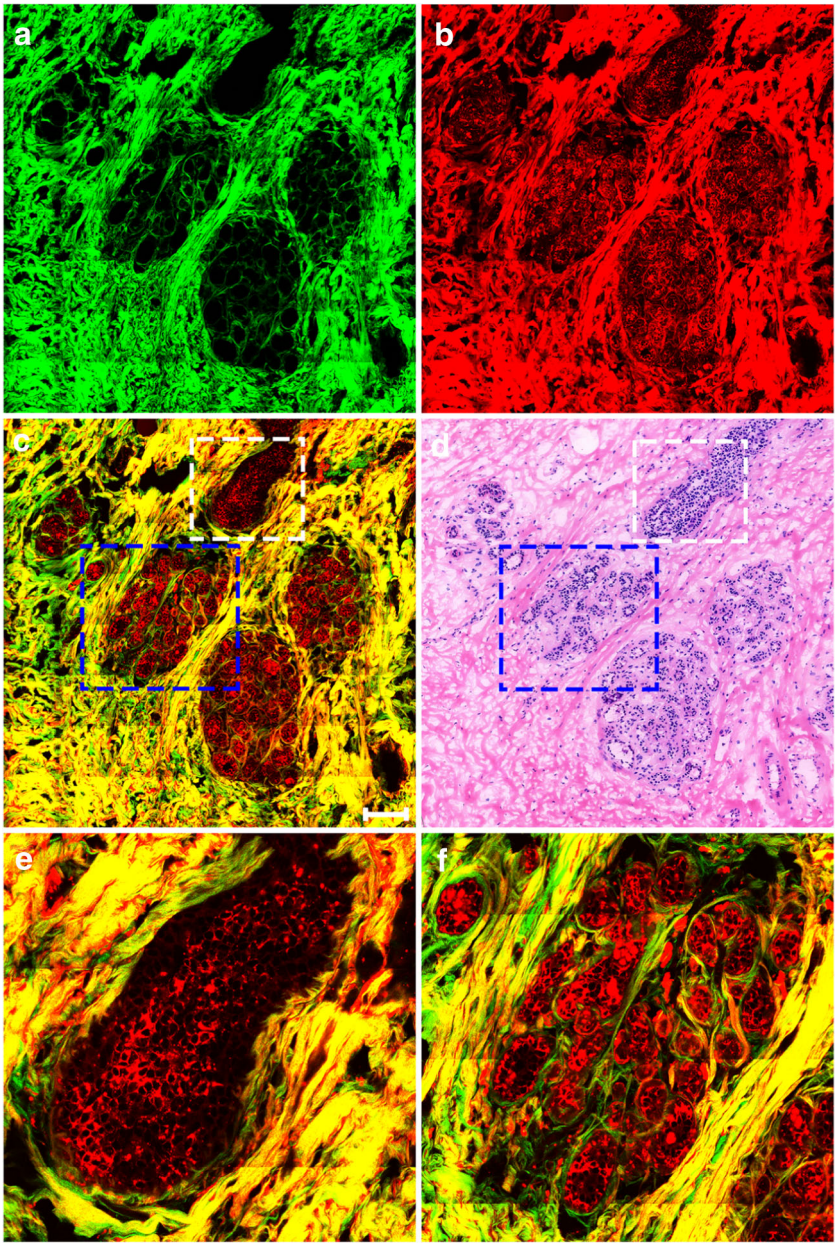

Fig. 1 Representative multiphoton images of normal breast tissue and corresponding H&E-stained image. Scale bar 100 μm. **a** SHG image. **b** TPEF image. **c** Overlay image. **d** H&E-stained image. **e**–**f** Magnification multiphoton images of the regions of interest (white box and blue box in **c**, respectively)

respectively. As presented in Fig. 4, the nuclear area from the post-treatment breast cancer tissues is obviously greater than that from the pre-treatment tumor tissues because neoadjuvant chemotherapy would induce nuclear enlargement. Specifically, the nuclear area from the tumor tissues following primary chemotherapy is 69.05 ± 31.77 μm^2; however, from the tumor tissues without therapy is 38.40 ± 12.34 μm^2. Large standard deviation indicates that nuclear pleomorphism is more pronounced in the breast cancer tissues treated with chemotherapy. Statistically significant difference ($P < 0.01$) in the nuclear areas was also found between the pre-treatment and post-treatment mammary carcinoma. Our data analysis suggests that this optical marker may help further differentiate the post-treatment residual tumors from the breast tumors without therapy.

Discussion

Breast cancer is a common form of malignant tumors in women and has a worse prognosis. Because neoadjuvant chemotherapy significantly improves outcome compared to surgery alone, it is increasingly applied to the treatment of breast cancer [21, 22]. Previous studies have also shown that presurgical therapy can reduce the volume of large primary breast cancer, thereby allowing successful removal of initially unresectable tumors, and increasing the proportion of locally advanced breast cancer patients who receive breast preservation surgery rather than mastectomy [23–25]. However, how to accurately detect residual tumor tissues, especially to identify residual tumor cells, is still an unsolved problem for the

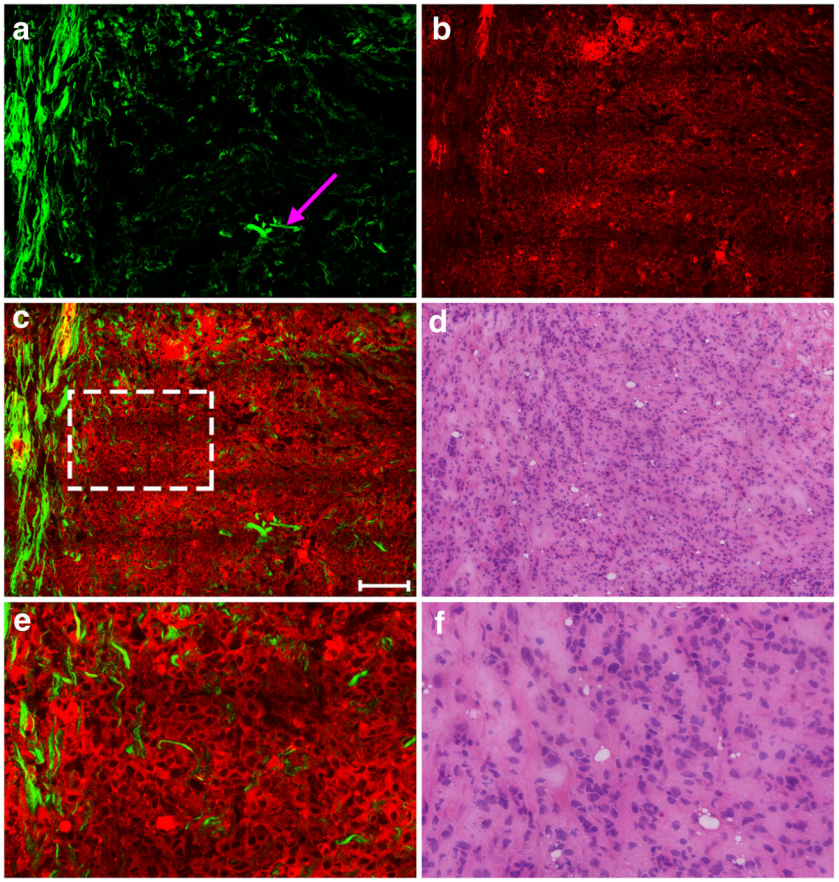

Fig. 2 Representative multiphoton images of invasive ductal carcinoma of the breast and corresponding H&E-stained images. Scale bar 100 μm. **a** SHG image. **b** TPEF image. **c** Overlay image. **d** H&E-stained image. **e**–**f** Zoom-in image of the boxed region in (**c**) and corresponding H&E-stained image. Pink arrow: a few fragmented collagen fibers

current medical imaging technology. Although histopathology analysis is always considered the gold standard in diagnosis, it requires time-consuming tissue processing; and more troubling, the diagnosis could only be made after surgery, which will affect the next step in developing treatment strategies. To overcome some of the defects associated with histopathologic processing, many researchers have been working to develop high-resolution imaging techniques.

Multiphoton imaging is an emerging imaging technology, which can directly image biological tissues with high-resolution and no marking, and is considered to be the most promising technology to realize optical biopsy [26, 27]. Some researchers have employed multiphoton microscopy to quantitatively assess the differences in redox ratio and collagen characteristics such as collagen density and orientation between normal breast and ductal carcinoma before and after preoperative treatment [28]. However, to date, few studies have explored the potential of MPM to detect residual breast tumors in patients after neoadjuvant treatment. Our experimental results show that TPEF imaging can label-freely identify residual malignancy after presurgical chemotherapy, particularly in patients who achieve a nearly complete remission and therefore have only small residual tumor cells, because there are strong endogenous fluorescence signals in cells such as nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD). Recently, a new portable intraoperative real-time multimodal label-free nonlinear imaging system has successfully visualized the tumor microenvironment of human breast tissue shortly after it was surgically removed from a patient in the operating room [29], and therefore was reported as bringing diagnostic potential into operating room. Once this technology is clinically available, it will help surgeons to accurately evaluate the surgical margin during operation, and thereby reducing recurrence rate and metastasis, and then achieving a better prognosis.

As breast matrix is rich in collagen fibers which have asymmetric central structure and can generate strong SHG signal, SHG imaging can monitor stromal changes better than histopathological examination and could provide more complementary information for clinical pathologists. Furthermore, this study also used a quantitative analysis of nuclear area to differentiate between the pre-treatment and post-treatment breast tumor cells. Statistical analysis demonstrates its correlation with the therapy, that is, the nuclear area of tumor cells after neoadjuvant chemotherapy is significantly larger than that before therapy. This conclusion is consistent with the results reported in previous studies [30–32]. It can be seen that changes in tumor nuclei can not only reflect the effectiveness of treatment, but also help to identify tumor cells left. In brief, MPM imaging allows in situ visualization of the suspicious lesions at cellular and subcellular levels, and therefore may

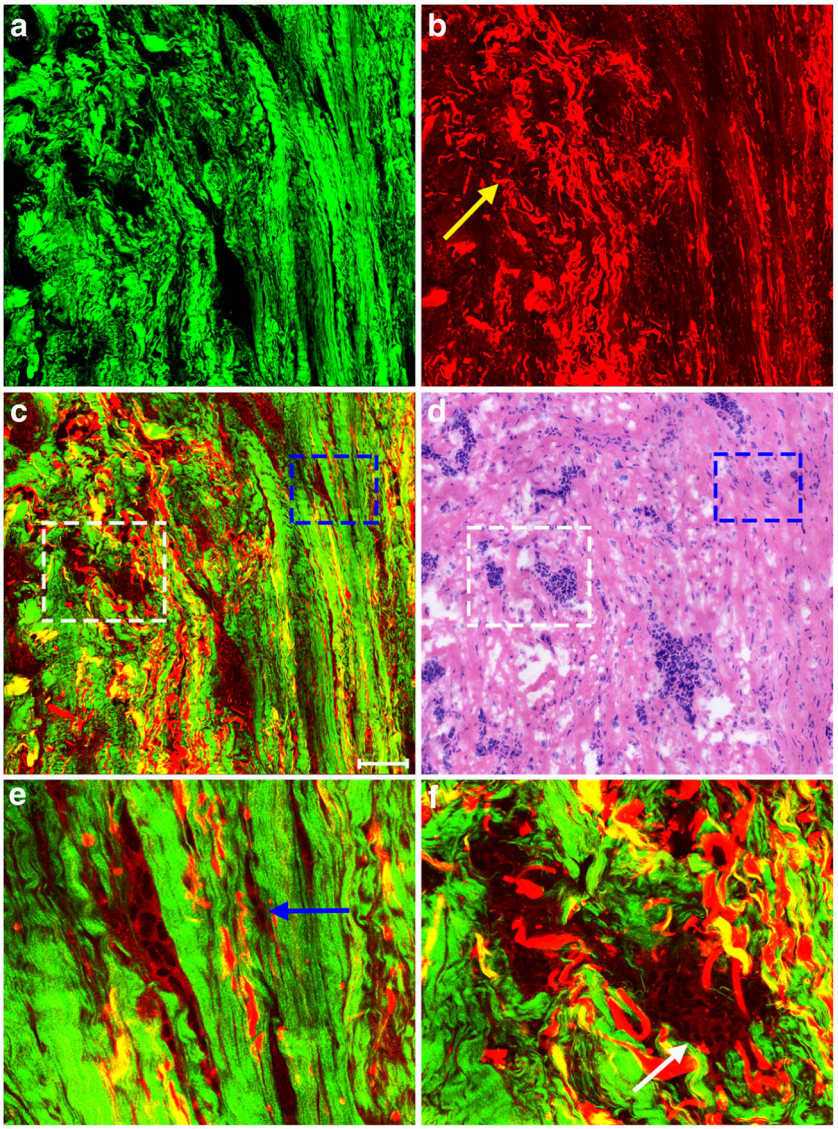

Fig. 3 Representative multiphoton images of invasive ductal carcinoma after neoadjuvant chemotherapy and corresponding H&E-stained image. Scale bar 100 μm. **a** SHG image. **b** TPEF image. **c** Overlay image. **d** H&E-stained image. **e**–**f** Magnification multiphoton images of the regions of interest (blue box and white box in **c**, respectively). Yellow arrow: some fragmented elastic fibers; blue arrow: residual breast tumor cells; white arrow: nested architecture of residual tumor cells with marked fibrotic reaction

help determine the surgical margin accurately in the future, followed by the resection of only the pathologic lesions, thereby minimizing patient morbidity associated with unnecessary excision.

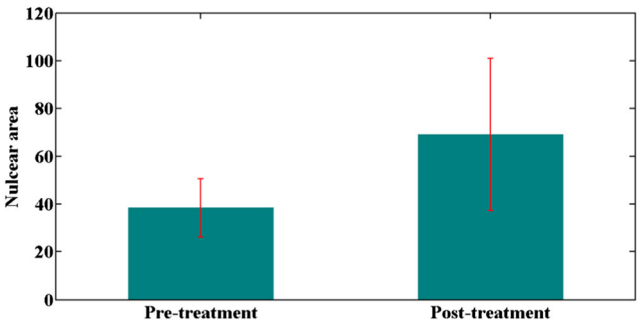

Fig. 4 Nuclear areas of the tumor cells from the pre-treatment and post-treatment invasive ductal carcinoma, respectively. Error bars indicate standard deviation

Conclusion

In this study, multiphoton microscopy was introduced to detect residual breast tumor cells after neoadjuvant chemotherapy. The results indicate that multiphoton images can not only directly identify residual tumor cells, but also provide quantitative diagnostic information. We have enough reason to believe that multiphoton microscopic imaging will be used for the detection of residual breast carcinoma with the development and improvement of this technique, and therefore could be used in the near future to aid in diagnostic decision-making.

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Compliance with ethical standards

Ethical approval All procedures performed in this study involving human participants were in accordance with the ethical standards of the Institutional Review Board of the Fujian Medical University Union Hospital.

Informed consent Every patient signed an informed consent before study participation.

Conflict of interest The authors declare that they have no conflict of interest.

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