Computer-aided Detection of Quantitative Signatures for Breast Fibroepithelial Tumors using Label-free Multi-Photon Imaging

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Abstract

Background

Fibroadenomas (FAs) and phyllodes tumors (PTs) are major benign breast tumors. They are pathologically classified as fibroepithelial tumors, composed of a proliferation of both epithelial and stroma. Although the clinical management of PTs differs from FAs, distinction by core needle biopsy diagnoses is still challenging. Computer-aided diagnosis is playing a pivotal role in accurate and objective evaluation of medical images. This technology opens up a new route to a solution for diagnostic problems.

Methods

A combined technique of label-free imaging with multi-photon microscopy and artificial intelligence was applied to detect quantitative signatures that differentiate fibroepithelial lesions. Multi-photon excited autofluorescence and second harmonic generation (SHG) signals were detected in tissue sections. A pixel-wise semantic segmentation method using a deep learning framework was used to separate epithelial and stromal regions automatically. Quantitative signatures, the epithelial to stromal area ratio, and the collagen SHG signal strength were investigated for their ability to distinguish between FA and PT lesions.

Results

Multi-photon microscopy recordings of tissue sections revealed distinct morphology between the epithelia and stroma, and further indicated that stromal regions emit a strong SHG signal which derives from collagen fibrils. However, this signal strength differs between the lesions, suggesting differences of collagenous molecular composition between the two lesions. In order to investigate hypertrophy of the stroma and compare this to the epithelial areas, an image segmentation analysis with a pixel-wise semantic segmentation framework using a deep convolutional neural network was performed. The deep learning-based analysis showed accurate separation of epithelial and stromal regions. Further investigation was conducted to determine if scoring the epithelial to stromal area ratio could be a marker for differentiating fibroadenoma and phyllodes tissues; we determined that most samples can be clearly separated but some are difficult to separate by the signature. Further investigations on the collagen SHG signal strength within the stromal area revealed accurate classification of the breast tissue lesions.

Conclusions

Molecular and morphological changes detected through the assistance of computational and label-free multi-photon imaging techniques enabled us to propose quantitative signatures for epithelial and stromal alterations in breast tissues.

Background

Fibroepithelial tumors of the breast are common benign lesions consisting of epithelial and stromal components. These include fibroadenomas (FAs) and phyllodes tumors (PTs), commonly seen in clinical practice. FAs arise from the epithelium and stroma of the terminal duct lobular unit. The histological hallmark is well-balanced epithelial and stromal proliferation. FAs are the most common benign breast lesions in young females. PTs were first fully characterized in 1838 by Muller [1], and are histologically characterized by epithelial-lined cleft-like spaces with hypercellular stroma, similar to a leaf in architecture. PTs include sub-classifications such as benign, borderline, and malignant, according to the WHO classification [2]. The distinction among the three subgroups is based on the combination of several histologic features. Benign PTs sometimes relapse with higher proliferative activity and may progress to borderline PT or malignant PT. Moreover, benign PTs mimic FAs in histopathology; therefore, both lesions are difficult to distinguish with core needle biopsy (CNB) or vacuum-assisted biopsy (VAB), despite different clinical courses. Almost all patients diagnosed with FA are recommended for follow-up, but surgery may be considered when they exhibit rapid growth or a size greater than 3 cm in imaging. On the other hand, once a patient is diagnosed with a PT by biopsy, regardless the size, wide excision with surgical margins ≥ 1 cm is recommended because of local recurrence [3]. Several studies have tried to find the clinical and histological factors to differentiate PT from FA, but these factors often overlapped. It was reported that the sensitivity of imaging and CNB for diagnosing PTs is 65% and 63% [4], and another report showed that the inter-observer variation was high with CNB when diagnosing fibroepithelial tumors [5]. Moreover, the upstaging to PT from fibroepithelial tumors diagnosed by CNB is often experienced in clinical practice. It was reported that the upstage rate was 37.5% for benign or borderline PTs in excised fibroepithelial tumors [6]. Stromal mitosis might be helpful for differential diagnosis of PT. Finding 2 or more stromal mitoses per 10 HPFs in the CNB specimen may indicate PT [7]. Immunohistochemical studies are also useful for differential diagnosis of fibroepithelial tumors, such as Ki67 or topoisomerase α [7]. Nonetheless, a report showed the mitotic counts and these immunostaining findings on CNB materials overlapped between FAs and PTs [8]. Thus, there is no clear definable cut-off of these histological or immunohistochemical features. Tumor heterogeneities result in both the difficulty and the interobserver variation in the diagnosis of fibroepithelial tumors. Therefore, although there are several predictive factors [3, 9–11], distinction between benign PTs and FAs still remains a difficult task, and hence stands as a diagnostic challenge to decide clinical management.

A label-free imaging technique with multi-photon microscopy (MPM), which enables high resolution fluorescence imaging, is attracting much attention as a histopathological diagnostic tool for assessing disease states. This technique has been extensively used for assessment of various diseases, including cancer and fibrosis [12, 13]. The near-infrared beam used for multi-photon excitation can excite endogenous fluorophores which include nicotine amide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD), elastic fibers, vitamins, and other metabolites [14]. In breast cancer tissues, this autofluorescence (AF) has been used to estimate cellular redox states as well as for assessment of cancers [15, 16]. At the same time, second harmonic generation (SHG) imaging is possible by MPM, which allows direct visualization of molecules possessing non-centrosymmetric molecules such as collagen. SHG imaging played vital roles for evaluating changes in fibrillar organization [17]. Combined

SHG and AF imaging was used for diagnosing breast cancers [15, 16, 18–21]. On the other hand, there have been few reported studies which surveyed the relation between benign breast lesions and MPM. Two subtypes of fibroadenomas were investigated using AF and SHG signals [22], and it was reported that measurement of collagen density by SHG imaging is useful for differential diagnosis of breast FAs and PTs [23]. This study simply investigated amounts of the collagen fibrils by quantifying the SHG signals as a potential diagnostic index. Although this achieved over 85% sensitivity and specificity, for more accurate detection, structural information regarding collagenous density and proliferation of lactiferous ducts should be included since fibroepithelial tumors were composed of the proliferation of the stromal and epithelial elements. Therefore, in order to refine signatures that differentiate the lesions more precisely, we took advantage of a machine learning-based approach for quantitative feature detection. Digital pathology is a field of computer-aided detection and evaluation of diseases, aiming to automate assessment. With the advancement of artificial intelligence, this approach is becoming more common in clinical investigations. In this study, we aimed to find novel morphological signatures of the FA and PT fibroepithelial lesions in breast tissues. To investigate whether morphological features which reflect stromal hypertrophy and epithelial proliferation can differentiate the lesions, we performed a pixel-wise semantic segmentation method using a deep learning framework. Accurate separation of epithelial and stromal regions allowed for estimation of the balance of epithelial to stromal regions, which can be a signature for differentiating FAs and PTs. Furthermore, combining this with the collagen SHG signal strength led us to finding a refined index to distinguish FA and PT lesions.

Methods

Patient-derived samples

The specimens were obtained from 10 female patients who were diagnosed with breast fibroepithelial tumor by core needle biopsy (CNB) or vacuum-assisted biopsy (VAB). All patients underwent lumpectomy or mastectomy between January 2012 and July 2018 and were pathologically diagnosed with FA or PT.

Preparation of tissue sections

The biopsy samples were fixed with 10% neutral buffered formalin for 24 h at room temperature and were subjected to embedding in paraffin. Sections 5 μ m thick were cut and stained with hematoxylin-eosin (HE) and picro-sirius red (PSR). PSR staining was performed using picro-sirius red stain kit (Polysciences, Inc.), which stains type I and type III collagens. Bright field images of the sections were acquired using a wide field inverted microscope (All-in-one fluorescence microscope BZ-X700, Keyence, Inc.) with a 20 $\times$ magnification objective lens (PlanFluor 20 $\times$ NA:0.45, Nikon, Inc.).

Image acquisition by multi-photon microscopy

We utilized an upright MPM (A1R-MP, Nikon, Inc.) equipped with a water immersion lens (CFI75 Apo 25 $\times$ W MP, NA:1.1, Nikon, Inc.), and a Ti:sapphire laser oscillator system (MaiTai eHP, Spectra-Physics, Inc.) for observing SHG and AF signals as described previously [24, 25]. For the detection of SHG and AF signals,

we employed excitation wavelengths of 950 nm with emission filter sets including 1) the dichroic mirror (DM) 495 nm and the shortpass filter 492 nm, 2) DM 560 nm and bandpass filter 525/50 nm (center wavelength/bandwidth), and 3) DM 662 nm and bandpass filter 617/73 nm. The field of view (FoV) of the single images was 0.5 mm × 0.5 mm and the resolution was 512 × 512 pixel, (i.e. the pixel size was 1 μm). Larger FoV images (whole tissues and 1 mm × 1 mm FoV) were obtained by stitching the single images. The images were originally recorded as 12-bit gray level images and were converted to 8-bit gray level images when analyzed computationally. For each patient sample, 6–12 regions were imaged and, in total, 33 and 43 images for FA and PT, respectively, were acquired.

Image segmentation by SegNet

The automated image segmentation of MPM images was performed using SegNet, a deep convolutional neural network architecture for multi-class pixel-wise segmentation [26]. For this supervised learning based image segmentation, we first prepared the training image data sets manually (ground truth). These data are a set of labeled (multi-level) images which were composed of three kinds of regions, “Epithelial,” and “Stroma,” and “Outer” regions. By comparing with the HE and PSR stained section images, any pixels of MPM images were classified into the three categories using area selection tools in Fiji (Image J) software. “Epithelial” regions include ductal epithelial cells and duct, while “Stroma” regions include collagen-rich stromal regions without any epithelial structure. “Outer” regions were outside of biopsied tissues. For training the SegNet network, 50% or 20% of randomly selected images of a total of 76 images were used, while the remaining images were used for the validation test. The learning parameters were as follows: Epoch number 5000, Mini batch size 4. In order to evaluate segmentation performance, we examined the total accuracy and the intersection of union (IoU) between the predicted and ground-truth images. The calculations for image segmentation were performed using the software MATLAB (Mathworks, Inc.).

Statistical analysis

Non-parametric statistical test was performed by the Kolmogorov-Smirnov test with a p-value < 0.05.

Results

MPM imaging characterizes morphological distinctions between epithelial and stromal regions for FA and PT lesions

We first summarized statistics of five FA and five PT patient samples which were subjected to the analyses (Table 1). The median sizes for FA and PT on the pre-operative imaging of ultrasound sonography were 3.0 cm (IQR 3.0-3.1 cm) and 2.9 cm (IQR 1.4-3.5 cm), respectively. For biopsy, CNB or VAB were used. The median numbers of biopsied specimens were 3 in both the FA and PT groups. In the FA group, two patients (20%) could not pre-operatively be diagnosed with FA or PT lesions. On the other

hands, in the PT group, two patients (20%) were pre-operatively diagnosed with FA. All patients underwent lumpectomy or mastectomy, and were finally diagnosed with FA or PT.

In order to examine how MPM images of SHG and AF signals characterize the morphological differences between epithelial and stromal regions in breast mammary gland tissues, we first performed MPM observation and histological analyses of tissue sections (Figure 1, Supplementary Figure 2 and 3). The histological examination was performed using HE and PSR staining method. Compared with the histological slices, the epithelial cellular structures are featured as slightly dark regions in the SHG images and the boundary between epithelia and stroma in the AF images can be recognized. On the places corresponding to the epithelia, cell nuclei were stained in the histological slices, indicating that these included mammary duct epithelia and lumens. Collagen-rich stromal areas were recognized as strong SHG signal areas in MPM images and regions stained red in the PSR staining images. Since PSR specifically stained collagen type I and III, the shapes and patterns of fibril structures were close to those observed in the SHG images, consistent with the SHG-illuminated collagen molecules. These results indicated that MPM images enabled us to morphologically differentiate epithelial and stromal tissues in breast tissues of fibroepithelial lesions.

Deep learning-based image segmentation approach for differentiating epithelial and stromal morphologies

It has been partly reported that FA grows more in stromal regions than PT. Therefore, for establishing a quantitative criterion for differentiating FA and PT, we attempted to score the epithelial to stromal area ratio. Thus, for automated quantification of these features, we took advantage of an image segmentation approach. In order to perform image segmentation, we employed the SegNet, a deep convolutional neural network architecture for semantic segmentation. This deep-learning based framework was shown to be high performance architecture for generic scene semantic segmentation. Thus, to implement the supervised image segmentation, we first prepared ground-truth image sets, namely, labeled images with three types of categorized regions, 'Epithelial', and 'Stroma', and 'Outer' regions (Figure 2). We manually selected these regions by comparing MPM images with the HE and PSR stained images. 'Epithelial' regions include ductal epithelial cells and ducts, while 'Stroma' regions include collagen-rich stromal regions without any epithelial structure. 'Outer' regions were outside of biopsied tissues. These were used for training and to test for the supervised machine learning approach. We first trained the network of SegNet using 50% of randomly selected images (38 images) for a total of 76 images. The remaining images were used for a validation test. Figure 3A showed the segmentation results. It seemed that segmentation results showed good performance both for FA and PT images. Differences indicated by magenta or green in the images were not significantly different. For the test data, the differences became slightly larger compared with the training ones (Figure 3B). However, the absolute differences still remained small. In order to evaluate the segmentation performance quantitatively, we examined the total accuracy and the intersection of union (IoU) between the predicted and ground-truth images (Figure 3C). The total accuracy for test image sets is ~94%, and the IoU for those is ~90%, indicating high segmentation performance. We further investigated performance results when the number of training

images decreased. We examined the case in which 20% of randomly selected images (15 images) were used for training data. The predictive algorithm still showed good performance (the total accuracy ~91%, the IoU ~86%), indicating that a small training set is sufficient for accurate results (Figure S4).

Computer-assisted scoring helps to diagnose FA and PT lesions

Based on the result of the image segmentation analysis, we performed scoring of the epithelial to stromal area ratio. The ratio in PTs were higher than FAs, because the leaf-like architecture was reflected the epithelial area including the lumen. We evaluated this score for each image and calculated its statistics (average $\pm$ standard deviation) for both ground-truth and predicted image data (Figure 4A). Both data showed that the score for PT is higher than that for FA. Therefore, the score can be an index to classify fibroepithelial lesions. The scores calculated using the ground-truth and predicted data showed almost the same values, suggesting that AI-based segmentation using supervised image data sets can return accurate signatures for diagnosis.

We looked for another quantitative feature for fibroepithelial tumor differentiation. Carefully looking at the SHG images, stroma in FA samples emit slightly stronger SHG signals than those in PT samples (Figure 1, Supplementary Figure 2 and 3). To investigate these points, we quantified the SHG image intensities within the stromal regions (Figure 4B). The averaged signal intensities showed that the FA sections emit stronger signals than the PT sections. PSR staining sections show similar extracellular collagen deposition, thus this suggested different collagenous patterns between FA and PT lesions, demonstrating an advantage of MPM for evaluating tissue samples. We combined the scores of epithelial to stroma area ratio and the SHG signal intensity within the stromal area. Two-dimensional scatter plots showed a clear separation of FA and PT samples. Although these indicated that individual values of the epithelial to stromal area ratio showed some mixture of the two lesions (Figure 5A and 5B), the SHG signal intensity clearly differentiated the two lesions. To computationally confirm this point, we performed linear discriminant analysis of these data. The results showed high accuracy of differentiation (Figure S5). The lines separating two lesions were almost perpendicular to the axis of SHG signal intensity, suggesting that SHG intensity is highly accurate in diagnosing features for fibroepithelial lesions.

Discussion

FA and PT are fibroepithelial tumors and consist of proliferation of both epithelial and stroma. FAs are concurrent proliferation of glandular and stromal elements. The stroma usually is hypocellular and may be fibrous, myxoid, or hyalinized. There is no stromal atypia and few mitotic activities. On the other hand, PTs are hypercellular fibroepithelial tumors characterized by an exaggerated stromal growth pattern with a leaf-like architecture. The leaf-like architecture is the elongated epithelial-lined clefts, resulting from stromal overgrowth just below the epithelium. Accurate evaluation of this morphological feature raise possibility of distinction of benign breast tumors. In this study, we highlight this point with help of MPM and a machine learning tool.

Label-free imaging using MPM enables us to observe unstained samples using endogenous sources of nonlinear signals and to diagnose several types of disorders. In breast tissues, fibrosis assessment based on the SHG signal, which comes from collagen molecules upon two-photon excitation, has been investigated [23]. Changes in collagen architecture in breast lesions can be observed. Therefore, SHG has been used to quantitatively characterize fibrillar collagen deposition. Furthermore, the strength of the SHG signal correlates with the molecular organization of living tissues. We actually obtained different signal levels of SHG intensity for FA and PT tissues, even though the histological staining results did not show clear differences between the two tissue images. This suggested that the two lesions show different ways of collagen deposition and different steric architecture of collagen fibrils. Further studies on these mechanical insights of how collagen accumulated in the fibroepithelial lesions are required. On the other hand, strong native fluorescence was emitted in the breast tissues. This included NADH, flavins, and vitamins [12–14]. We used an excitation laser at a 950 nm wavelength for MPM image acquisition, in which the primary intracellular sources of fluorescence in liver tissues are NADH and flavins. NADH and flavins allowed us to visualize epithelial and stromal morphology and to discuss histological characteristics. An advantage of using MPM is to estimate quantitative features, such as fluorescent and SHG signal intensities. Here, we evaluated SHG signal intensities which were not affected by photo-bleaching like fluorescence. In addition, these methods do not show inter-assay differences such as staining variability, which leads to descriptive and semi-quantitative evaluations arising from observer discrepancies.

We evaluated SHG signal intensity and the epithelial and stromal area ratio. To proceed with the computer-assisted diagnosis method, we used SegNet, a deep convolutional neural network-based image segmentation tool. SegNet returns high accuracy of predicted image data. We first tried 50% of total image for training data. This resulted in over 90% coincidence between the ground-truth and predicted data. Next, we reduced the number of images used for training to 20% of total images. This also showed 90% accuracy. This means that larger numbers of images were not required and suggests that the method can be applied easily to new images once a reliable data set is constructed. The two types of scores were useful to differentiate FA and PT lesions. Scatter plots and discrimination analysis revealed that the combination of the two scores is essential for individual classification. The SHG intensity reflected the molecular organization of collagens, thus MPM has an essential role in diagnosing fibroepithelial lesions. Although deep learning costs computational demand, once the neural network that returns reliable results was constructed, the image segmentation applied to the test data was rapidly obtained. Thus, through fixing the acquisition conditions, we can realize a computer-assisted objective diagnostic method for detecting breast lesions.

Conclusions

In order to establish a method for scoring fibroepithelial lesions in breast tissues, we used multi-photon excitation microscopy and computational image analyses. Deep learning-based image segmentation is useful for differentiating epithelial and stromal regions in the lesions. We evaluated the potential utility of scoring methods for classifying fibroepithelial tumors in breast tissues. We showed that the combined

features of SHG intensity and epithelial and stromal area ratio accurately differentiated diseased tissue images. Therefore, a proposed method of computer-guided diagnosis would provide a promising approach for morphological- and molecular-based diagnosis of breast tumors.

Abbreviations

FA: Fibroadenoma

PT: Phyllodes tumor

CNB: Core needle biopsy

VAB: Vacuum assisted biopsy

HE: Hematoxylin-eosin

PSR: Picro-sirius red

MPM: Multi-photon microscopy

DM: Dichroic mirror

FoV: Field of view

AF: Autofluorescence

SHG: Second Harmonic Generation

NADH: Nicotine amide adenine dinucleotide

FAD: Flavin adenine dinucleotide

AI: Artificial Intelligence

IoU: Intersection of union

Declarations

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

K.T. performed experiments. T.S. performed digital image analysis. K.T., T.S., Y.K., and T.I. wrote the manuscript. A.M., K.N., E.K., H.N., R.A. and M.Y. collected the clinical and pathological data. Y.K., R.K., and Y.T. contributed to the concept and helped to write the manuscript. All authors reviewed and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

This survey was approved by the Institutional Review Board of the Ehime University School of Medicine (approval number 1807005). Core needle biopsies or vacuumed assisted biopsies were performed under sonographic guidance by breast surgeon at the breast center of the Ehime University.

Consent for publication

Not applicable

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Table

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

Figure 1

Image comparisons of the FA and PT lesions. Serial section images of multi-photon microscopy (MPM) and histological sections of HE and PSR staining for the FA (A) and PT (B) lesion. MPM images include

the SHG signal indicated in blue and autofluorescence signal indicated in green and red. PSR stained collagen type I and III in red, and cell cytoplasm in light yellow. Scale bar, 100 μm .

Figure 2

Schematic of quantification strategy for breast fibroepithelial lesions based on the multi-photon microscopy (MPM) image. Areas surrounded by yellow dotted lines denote lactiferous duct epithelia and lumens, while areas outside of those areas denote stroma. All images acquired by MPM was subjected to manual segmentation to construct ground-truth image sets for automated image analysis. Using ground-truth image sets as training image data, supervised machine learning of pixel-wise image segmentation was performed, which assigned all pixels to the epithelial, stromal, or outer area. On the basis of the segmented image sets, measurement of SHG intensity within the stromal area and scoring lateral duct epithelial to stromal area were performed.

Figure 3

Results of image segmentation by a deep learning based framework, SegNet. (A) Results of training image sets. Original multi-photon microscopy images, ground-truth images, predicted images, and difference images were shown from left to right for both FA and PT images. Differences in images indicated FN areas as magenta and FP areas as green. (B) Results of test image sets. (C) Numerical evaluation of the segmentation results. The total accuracy between the ground-truth and predicted

images and the weighted IoU, which indicates the area weighting sum of each IoU value, are shown for training and test data sets. The bar denotes average; the error bar denotes standard deviation over the data calculated from image sets.

Figure 4

Quantification results of multi-photon microscopy images for breast fibroepithelial lesions. (A) Averaged SHG signal intensity within the stromal area for FA and PT lesions. (B) Epithelial to stromal area ratio for FA and PT lesions. Scores calculated using the ground-truth data and predicted data are shown. The bar denotes average; the error bar denotes standard deviation over the data calculated from image sets.

Figure 5

Scatter plots of the two quantification scores. (A) Scatter plot for the ground-truth data. (B) Scatter plot of the predicted data. The filled and open circles denote FA and PT data, respectively. The same color represents samples derived from the same patient.

Supplementary Files

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