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ORIGINAL PAPER

Analysis of Cancer Marker in Tissues with Hadamard Transform Fluorescence Spectral Microscopic Imaging

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Abstract Quantum dots (QDs) probes were used to tag and trace cancer biomarkers in cancer tissues based on the system of home-made Hadamard transform (HT) spectral microscopic imaging, which can be applied to provide high-resolution fluorescence spectrum and image of single cells and tissues. In situ fluorescence imaging for cancer marker proteins, such as estrogen receptor (ER), human epidermal growth factor receptor 2 (HER2), proliferating cell nuclear antigen (PCNA) and cytokeratin 20 (CK20) in tumor tissues, were realized by using the HT system to capture quantitative information for these proteins when tumor tissues were immunostained with QDs probes. A method to evaluate tumor malignancy of the specimens based on in situ analysis of distribution of marker proteins was proposed based on the comparative study of positive samples and negative controls. The investigation of ER contents of the cores in breast cancer tissue microarrays (TMAs) shows that the technique of QDs-immunohistochemistry (IHC)/HT spectral imaging is more sensitive than conventional IHC method. The results also demonstrate that the QDs-IHC/HT spectral imaging technique can be applied to visualize and quantitatively measure the subcellular molecules inside tumor tissues, and the coupling of HT spectral imaging to the probing of subcellular molecules with QDs has great potential in biology and medical diagnosis.

Keywords Cancer biomarkers · Estrogen receptor · Quantum dots · Fluorescence imaging · Spectral imaging · Hadamard transform

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Introduction

Some biomacromolecules are closely associated tumorigenesis and growth process, so the targets of them in cancer cells are named cancer markers, which are very important in clinical treatment and cancer research. Estrogen is implicated in the inception and progression of breast cancer, which is known to be the most frequent form of cancer in women in the world. The effects of estrogen in human breast tissue are mediated mainly by estrogen receptor (ER) and ER also mediates the effects of therapeutic endocrine agents in breast cancer, and its presence indicates a greater likelihood of response to this form of treatment [1]. The molecule-targeted therapy for human epidermal growth factor receptor 2 (HER2) has a tremendous impact on the treatment of breast cancer [2]. HER2 over-expressed in approximately 25~30 % of breast cancer and plays an important role in breast cancer prognosis

and treatment selection [3]. Therefore, HER2 status is now an integral part of the clinicopathological practice of breast cancer [4, 5]. Proliferating cell nuclear antigen (PCNA) acts as a process factor for DNA polymerase delta in eukaryotic cells, which is important for both DNA synthesis and DNA repairing [6, 7]. PCNA is over-expressed in proliferative cells and objectively reflects the proliferative activity of tumor cells. Therefore, PCNA is widely used in prognosis and diagnosing the proliferation activity of tumor cell, especially in breast cancer [8, 9]. Cytokeratins (CK) are proteins of keratin found in the intracytoplasmic cytoskeleton of epithelial tissues [10]. CK20 is commonly expressed in the gastrointestinal tract. In surgical pathology, the immunoassays of cytokeratins are widely used for various epithelial tumor diagnosis and characterization [11].

Immunohistochemistry (IHC) analysis of the cancer marker proteins have been widely applied in clinical cancer diagnosis, and quantum dots (QDs)-based immunofluorescent nanotechnology (QDs-IHC) for molecular pathology has potential advantages in delineating the cancer heterogeneity [12]. Quantum dots (QDs) have been widely applied as a fluorescence label for biomedical analysis [13]. QDs-IHC determination provided a more sensitive method than conventional IHC techniques for detection of HER2 in clinical breast cancer diagnosis [14]. Several photo-physical advantages of QDs, such as wide excitation spectrum, narrow fluorescence emission peak, multiple colors with a single excitation, high photostability and long fluorescence lifetime, make QDs fit very well for the fluorescence spectral imaging analysis in biomedical applications [15].

Hadamard transform (HT), which is based on the mathematic transforms of square waves and can be understood as the weighing design principium of statistics applied in optical applications, is one of the most important spectroscopic modulation techniques [16]. Hadamard multiplexing is well suited to multiplexed imaging when it is performed by a Hadamard mask, which is a multi-slit array, and the slits are made transparent or opaque. Twenty years ago, Morris's group applied a variety of HT systems to generate transverse photothermal deflection images and Raman images using movable masks [17–20]. In our previous reports, a system of HT microscopic fluorescence imaging which can generate 511×512 pixel image was developed using a movable mask and the system was applied to quantify the DNA content in single cells labeled with fluorescent dyes [21]. More recently, we developed a HT spectral imaging microscope and it was successfully applied to capture four-dimensional (4D) images for samples-location coordinate (X and Y), fluorescence intensity (Z) and wavelength or time to probe important intrinsic or extrinsic biomolecules in cells or tissues [15], and this method has been used in the quantitative evaluation of the efficiency the different cancer marker probes [22, 23], the results demonstrate that in situ quantitative evaluation of cancer markers is feasible.

In this report, QDs at different emissions are used to probe the four important cancer biomarkers ER, HER2, CK20 and PCNA in cancer tissues. Based on the visualization and spectral analysis of the marker proteins with the home-made HT spectral imaging microscope, in situ fluorescence imaging to evaluate tumor malignancy for breast tissues are discussed to show the potential of HT spectral imaging system in biomedicine. The data for the expression level of ER from TMAs and clinical tissues obtained with HT system show the high sensitivity and specificity of this technique in this field.

Materials and Methods

Materials

All tissue specimens were obtained from Hubei Key Laboratory of Tumor Biological Behaviors and Zhongnan Hospital of Wuhan University. The specimens include breast cancer and gastric cancer tissues, normal breast and gastric tissues. Normal breast and gastric tissues were used as negative control for the two cancer specimens, respectively. The specimens were fixed with formalin and embedded in paraffin (formalin-fixed and paraffin-embedded, or FFPE) and cut into 4 μm sections for QDs-IHC profiling.

Breast cancer tissue microarrays (TMAs) were obtained from Guilin Fanpu Biotech, China (Lot number: BCR1503), with 150 cores of breast specimens involved 75 cases, from the patients whose ages were between 25 and 86 years old (median 50 years old). TMAs were used for both QDs-IHC and conventional IHC staining.

The streptavidin(SA) coupled CdSe/ZnS QDs(595 nm) were provided by Wuhan Jiayuan Quantum Dots Co., Ltd. The QDs labeling operation is the same as our previous report [14, 24]. QDs are coupled to each marker protein indirectly through the biotin-SA system. Primary antibodies, mouse anti-human PCNA antibody, rabbit anti-human ER antibody, mouse anti-human CK antibody and mouse anti-human HER2 antibody were used to connect PCNA/ER/CK/HER2 in specimens. Biotin tagged IgG was used to connect the SA-QDs and the primary antibody. The negative control tissues were operated with the same processes.

Apparatus

The details of the home-made HT fluorescence spectral imaging microscope have been introduced in our previous work [15]. A defocused argon ion laser (35LAP 431, Melles Griot, USA) at 454 nm (4 mW output power, 4.2×10^3 W/m^2) was applied to excite the QDs labeled specimens for fluorescence imaging throughout the experiment. A 25×/NA 0.65 objective was used throughout the experiment to capture the HT fluorescence image or fluorescence spectrum for the tissue

sections. After bioconjugating operation, the maximal emission wavelength of the QDs might slightly change. In this case, HT imaging and fluorescence measurements were performed at the actual maximal emission wavelength.

The TMAs and tissues sections were also examined under Olympus BX51 fluorescence microscope equipped with an Olympus DP72 camera (Olympus Optical Co., Ltd., Tokyo, Japan). The QDs were excited by blue lights from the mercury lamp, and the digital images of QDs-IHC and conventional IHC staining were also captured by DP72 camera under the same conditions with a 10× objective, which could obtain the whole images for each core of breast cancer, making it more accurate and representative in tumor markers assay [25, 26].

Results and Discussions

Marker Protein Probing and HT Spectral Imaging

Figure 1 shows the comparison of breast cancer specimen (positive sample) and normal tissue (negative control), in which marker proteins are probed with CdSe/ZnS QDs emitting at 600 nm. The HT imaging technique can be used to directly show the distributions of the probed proteins. Figure 1a is the HT fluorescence image of ER and it clearly shows that ER locates in the nuclei. Similarly, HER2 and PCNA are observed in highly-malignant breast tumors and CK20 is also observed in highly-malignant gastric tumors. Figure 1a and c respectively show that both ER and PCNA are over-expressed in the nuclei, whereas Fig. 1b and d respectively show that HER2 and CK20 are over-expressed on cell membrane. In contrast, the specimen of negative control (Fig. 1e) shows no expression of the four proteins.

The spectrum of positive sample exhibits a strong peak about 595 nm, whereas the negative control shows no peak (Fig. 1f). Clearly, the fluorescence signal from the probed protein can be used as a proof for clinical examination because fluorescence intensity of the QDs can be used to quantify the amount of the marker proteins [27]. Similarly, HER2 and PCNA in breast tissues and CK20 in gastric tissues were probed with QDs based on the microscopic fluorescence spectra.

In conventional fluorescence imaging for tissues, autofluorescence is hardly neglectable. However, the HT spectral imaging system in this study is an excellent tool to overcome autofluorescence and images with high *S/N* ratio can be obtained. We found that autofluorescence of the QDs treated tumor specimens is much weaker than the fluorescently probed cellular chemicals and contributes no more than 1 % to the total signals. When the autofluorescence is considerable, a narrower entrance-slit of the spectrometer can be used to greatly reduce its contribution to the total signals and accordingly *S/N* ratio is improved.

Furthermore, an advantage of spectral imaging technique is using the spectral unmix method remove autofluorescence background based on the difference spectra of the probe, autofluorescence and probe staining tissue [28]. The HT spectral imaging system with spectral unmix method could get a "pure" image without autofluorescence background. Spectral imaging and spectral unmix is a universal method, which can be applied with organic fluorescence dyes, QDs, and even multiple IHC [29]. The QDs probe's stability is much better than organic fluorescence dyes, especially by laser exciting in our previous research [15], which is very important for measurement the fluorescence intensity for avoiding the adverse effects of photobleaching and obtaining repeatable results.

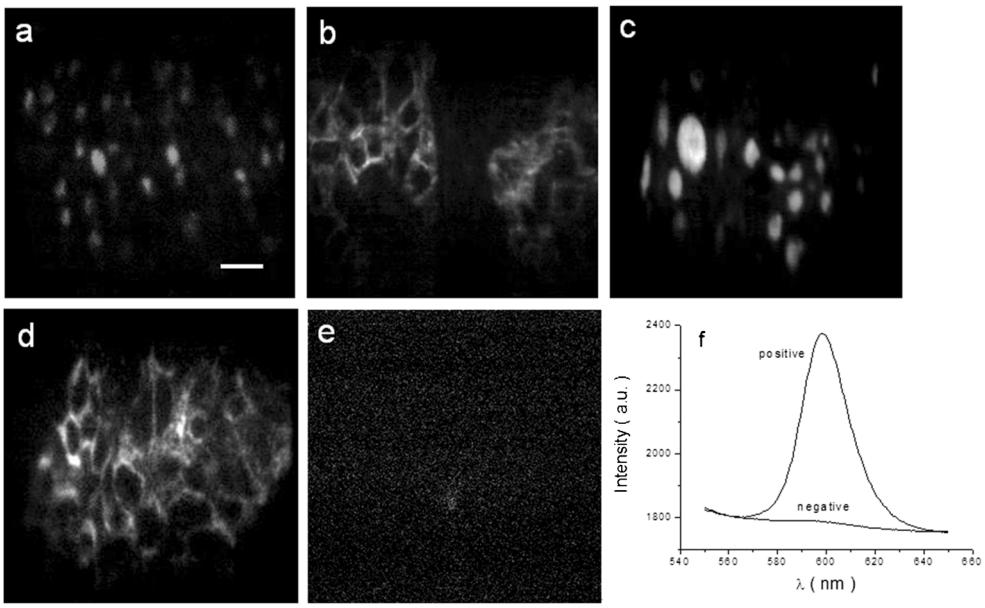

Fig. 1 600 nm QDs probing cancer biomarkers with Hadamard transform spectral imaging. **a** QDs identifying ER in breast cancer tissue; **b** QDs identifying HER2 in breast cancer tissue; **c** QDs identifying PCNA in breast cancer tissue; **d** QDs identifying CK20 in gastric cancer tissue; **e** negative control of (**a**)–(**d**); **f** microscopic fluorescence spectra of the ER positive and negative breast cancer specimen. Scale bar 20 μm

With the elimination of autofluorescence, high contrast HT fluorescence images for tissue sections can be obtained. Without the interference of autofluorescence, gray-level value of the pixels in the HT images can be used to describe the fluorescence emission intensity of the QDs probed marker proteins directly because the fluorescence intensity means the amount of the cancer markers, thereby the quantitative analysis of the marker proteins in cancer tissue is possible. Due to the over-expression of ER and PCNA in cell nuclei, the amount of marker protein in each nucleus was obtained through HT images. Figure 2 shows the distribution of PCNA contents of 23 cell nuclei in the same view-field (Fig. 1c). Clearly, the PCNA contents inside these nuclei vary dramatically and it implies that both the variation of the size and the rich amounts of PCNA demonstrating the highly proliferative activity of the cancer cells.

In conclusion, analysis of marker proteins based on spectral analysis can be applied for evaluation of tumor malignancy with high specificity.

Comparison of QDs-IHC and Conventional IHC Staining Analysis of ER

In this study, the comparison of QDs-IHC and conventional IHC staining analysis of ER was made with HT spectral imaging analysis. The ER intensity of breast tissue cores in TMAs was obtained from conventional IHC staining analysis and HT spectral imaging analysis of QDs-IHC staining. In contrast, QDs-IHC staining fluorescence imaging analysis of TMAs was applied. All measured samples were scored as integral numbers ranging from 0 to 4 and on a scale representing the estimated proportion and relative intensity of the positive-staining tumor cells. Figure 3a shows the comparison of the expression level of ER (scores) based on the measurements of 50 different positions of breast cancer cores

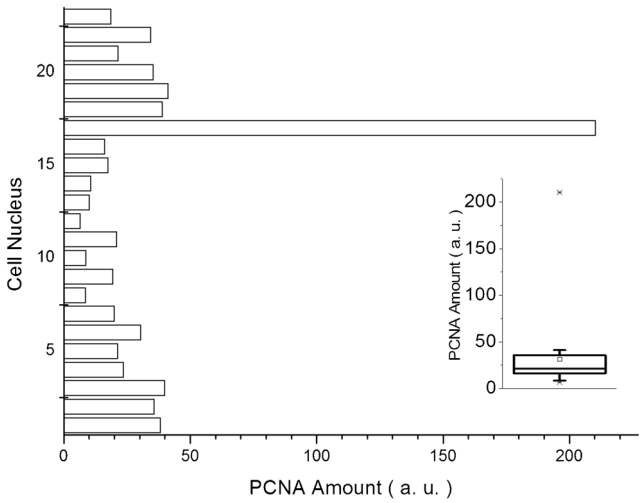

Fig. 2 PCNA contents and distribution in breast cancer cell nuclei, obtained from Fig 1c. The insert is the distribution of ER contents

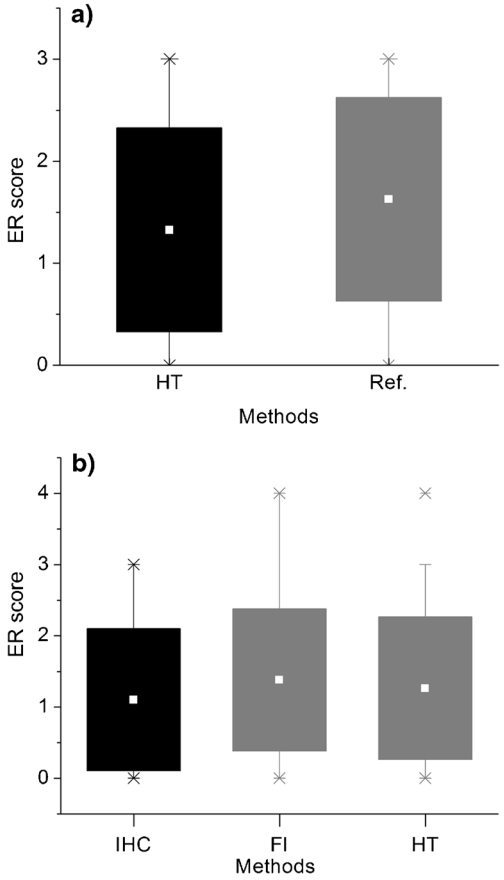

Fig. 3 Comparison of the measured ER levels (scores) in breast cancer core in TMAs by different methods. **a** HT spectral imaging analysis of QDs-IHC staining (HT) and the information supplied by the manufacturer (Ref.); **b** conventional IHC staining (IHC), fluorescence imaging analysis of QDs-IHC staining and HT. The data for (**a**) are based the measurements ER expression levels of 50 different positions of breast cancer cores in the same TMAs with three analytical methods: HT, IHC and FI

in the same TMAs by HT spectral imaging analysis of QDs-IHC staining (HT) and the information supplied by the manufacturer. Figure 3a shows that the two groups of data are highly accordant. This demonstrates that the HT spectral imaging analysis is accurate and reliable. The result of ER levels (scores) in 150 cases of breast tissue cores in TMAs obtained with the three methods: HT, IHC and fluorescence imaging (FI). Figure 3b reveals that there are no significant differences (P=0.095) in the ER expression levels between the data obtained from HT spectral imaging and from conventional IHC staining or conventional fluorescence image analysis based on QDs-IHC staining. Moreover, we found that the results of the three methods are very close when ER is in high levels, but in case of low ER intensities, the fluorescence digital image analysis of QDs-IHC staining is more sensitive than conventional IHC staining analysis, and HT spectral imaging analysis of QDs-IHC staining is the most sensitive. Among the three techniques, HT spectral imaging is most easily to detect weak signal of ER because autofluorescence from the sample is

greatly reduced in this case and highest sensitivity and specificity are shown.

ER Content Distribution Analysis of Breast Cancer Tissues

In this study, the ER content distributions of 36 breast cancer tissues were analyzed. ER in tissue sections was tagged by red QDs probes (595 nm). When red fluorescence from cell nuclei was observed under fluorescence microscopy and the fluorescence peak at 595 nm of QDs was exhibited in the obtained fluorescence spectra, we conclude that ER shows positive expression in this tissue, and ER content distribution of this tissue can be obtained by HT spectral imaging; in contrast, the tissues are ER-negative and the ER content distribution of these tissues cannot be obtained. The ER contents of breast tissues sections were obtained from the fluorescence intensity of 595 nm QDs by HT spectral imaging analysis. Figure 4 shows the distributions of ER contents from 36 breast cancer tissues, and at least 100 cells were counted for each tissue section.

The results are shown in a diamond box (Fig. 4). Half of the 36 tissues are ER- negative tissues, which ER contents only display a zero level line. In contrast, another half of the tissues are ER over-expressed and could be classified into two types by median line: some tissues show a rhombus of which the top greater than the bottom (type A) and some tissues show two symmetrical triangles beside on the median line (type B). ER distribution of the two types of tissues shows different ER expression mode, which may be ascribed to different subtypes and clinical stages of breast cancer. Type A means there are lots of high ER contents cells in this type tissue, and type B means ER contents close to a normal distribution and few high ER contents cells in this type tissue. In this case, the type A tissue is high ER active and maybe sensitive to control ER expression in clinical treatment. Therefore, the result can be used for the evaluation of tumor malignancy and clinical treatment process for cancers.

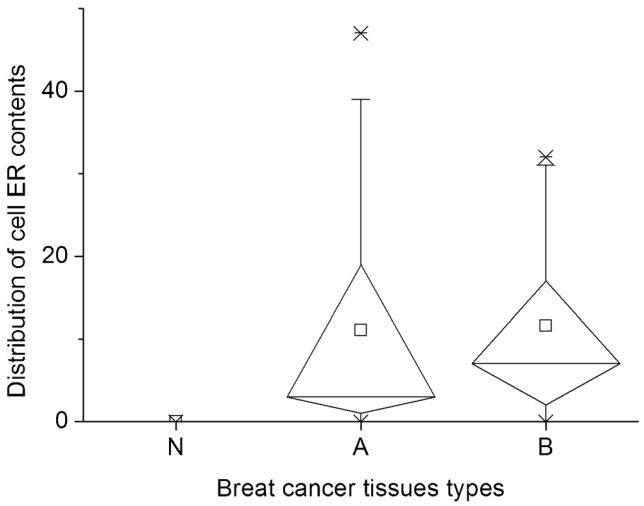

Fig. 4 Different distributions types of ER contents from 36 breast cancer tissues

Conclusions

The technique of HT spectral imaging analysis of cancer makers is very valuable to attain accurate and reliable results in biomedical analysis especially cancer diagnosis with high sensitivity and specificity. QDs are quite suitable to be applied as a fluorescence label for biomolecules in tissues probed with spectral imaging technology. Based on QDs immunostaining cancer marker proteins in cancer tissues, fluorescence spectra and spectral images were captured by the HT imaging microscope. Analysis of marker proteins based on spectral imaging can be applied for the tumor evaluation with higher specificity. The result of comparative studies of ER contents of the core in TMAs shows that QDs-IHC/HT spectral imaging analysis is more sensitive than conventional IHC analysis. The analysis of ER content distributions in breast cancer tissues shows that the ER content distributions may be classified into different types. In conclusion, the quantitative analysis of cancer makers based on HT spectral imaging analysis is more advantageous than classical method due to its high sensitivity, specificity and rich information.

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