Potential Clinical Applications of Next Generation Fluorescence Immunohistochemistry for Multiplexed and Quantitative Determination of Biomarker in Breast Cancer

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Abstract
Assessing breast cancer tissues for expression of multiple biomarkers, such as the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), provides critical information for patient management. However, the predictive power of these biomarkers for the selection of personalized therapeutic approaches is still limited. Application of fluorescence immunohistochemistry (IHC) has offered some promising breakthroughs in the multiplexed imaging and quantification of biomarker expression and may improve the sensitivity and reliability of clinical diagnostics. This Review discusses recent fluorescence IHC of biomarker in breast cancer tissue samples and compares this method with classic pathological methods of biomarker detection.

Keywords
Nanoparticle, Nanomedicine, Breast cancer, Pathology, Immunohistochemistry, Fluorescence, Quantum dot, Vectra

Abbreviations
HER2: human epidermal growth factor receptor 2; IHC: Immunohistochemistry; ER: estrogen receptor; PR: progesterone receptor; DAB: 3,3’-diaminobenzidine; FITC: fluorescein isothiocyanate; AQUA: automated quantitative analysis of histological sections; QD: quantum dot; FFPE: formalin-fixed paraffin-embedded.

Introduction
Clinical criteria, including immunohistochemical biomarkers, such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), are routinely employed for classification of breast cancer and to help determine the optimal approach for therapy; ER, PR and HER2 statuses help to determine the optimal regimen for endocrine therapy, chemotherapy, and anti-HER2 molecular-targeted therapy. HER2 have both prognostic significance and therapeutic value, especially for predicting response to anti-HER2 therapies, such as trastuzumab, lapatinib, pertuzumab, and trastuzumab emtansine [1-4]. The approach published by the St. Gallen Panels [5] is based on available clinicopathological testing; however, this classification approach has not been fully validated. Although, the classical immunohistochemistry (IHC) method, which uses 3,3’-diaminobenzidine (DAB), is widely used method of assessing these factor, it has some disadvantages, such as susceptibility to interfering factors, unstable sensitivity, high discrepancy among laboratories, subjective interpretation, and semiquantitative nature [6-8]. Therefore, a sensitive, accurate, and quantitative method for detection and measurement of these biomarkers is urgently required. To address these issues, organic dye- and fluorescence nanoparticle-based immunofluorescence techniques, whose properties are summarized in Table 1, are thought to be suitable for quantifying protein expression.

Here, we review on the recent applications of IHC for breast cancer pathology, particularly using fluorescence immunohistochemistry for multiplexed staining and quantitative analysis.

IHC modality for multiplexed

IHC with chromogen: IHC has been used for protein marker detection and localization in tissue specimens for decades [9]. IHC with DAB is the most conventional IHC protocol used today. IHC with DAB has several advantages over immunofluorescence stains, including that analysis can be performed using transmission light microscopy, and that tissue autofluorescence does not influence interpretation. Thus, the stain is long lasting and can be reviewed at a later time [10]. Diagnostic IHC has been improved by the development of companion diagnostics, such as HercepTest®[9], to predict responses to a specific therapy [11]. Computerized image analysis systems for pathology have been established [12,13], and other software algorithms have also been shown to provide data that correlate highly with pathology scores [14].

As a method of IHC with chromogens, Vectra® technology is promising, quantitating overlapping biomarkers using chromogenic multiplex immunohistochemistry in prostate cancer [15]. Biomarker expression analysis and tissue component segmentation

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was performed with Vectra® software (Nuance® software and inForm® software, respectively). In breast cancer, ERβ1 localization and quantitative expression were assessed in formalin-fixed, paraffin-embedded (FFPE) tissue sample using the Vectra® platform [16]. Although there are some limitations such as detection reliability of tissue and cell segmentation, this method promises an objective and reproducible approach for biomarker analysis.

For IHC with chromogens, there is a linear relationship between the amount of antigen and the staining intensity only at low levels [8]. And the intensity of DAB staining depends on the enzyme activity of horseradish peroxidase (HRP), so the staining intensity is significantly influenced by reaction time, temperature, and HRP substrate concentrations. Furthermore, the intensity of DAB staining often differs between experimenters [17]. Therefore, this method has some disadvantages, such as susceptibility to interfering factors, unstable sensitivity, discrepancy among laboratories, and subjective interpretation [18]. Thus there are some worries that they are not highly quantitative because these methods with chromogens.

**Fluorescence IHC by organic fluorescence dyes:** The fluorescent labeling of biomolecules using organic dyes, such as fluorescein isothiocyanate (FITC), DAPI, Cy-3, Cy-5, and rhodamine is widely employed in biological imaging and clinical diagnosis. A DeltaVision Restoration deconvolution microscope, combined with fluorescent IHC, has been used to obtain reproducible and quantitative estimates of ER and PR expression in FFPE tissue samples, with FITC, DAPI, and rhodamine as organic fluorescence dyes [19].

Camp and colleagues developed a system for compartmentalized, automated, quantitative analysis of histological sections (AQUA) [20]. For automated analysis, AQUA uses a modified IHC technique with Cy-5 tyramide. AQUA provides highly reproducible analysis of target protein expression with the use of a continuous scale, and preserves spatial information, including tissue and subcellular localization. AQUA was used to assess ER, PR, and HER2 protein expression in a large set of tissues in clinical trial enrollment [21]. Both ER and PR showed unimodal distributions and significantly predicted disease-free survival when tested as continuous variables and adjusted for node status, tumor size, treatment, and menopausal status. HER2, measured as a continuous variable, had a biphasic effect on disease-free survival. Patients with either high or low expression of HER2 exhibited worse outcomes than patients with intermediate HER2 expression.

**Table 1:** Comparison of the physical properties of fluorescence IHC. Organic fluorophores.QD, Avi-TMR.

<table>
<thead>
<tr>
<th>Property</th>
<th>Avi-TMR</th>
<th>Quantum dot</th>
<th>Organic fluorophores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorption spectra</td>
<td>Similar to their emission spectra</td>
<td>Broad spectra with size dependent excitation peak</td>
<td>Similar to their emission spectra</td>
</tr>
<tr>
<td>Emission spectra</td>
<td>Broad</td>
<td>Narrow</td>
<td>Broad</td>
</tr>
<tr>
<td>Photobleaching</td>
<td>High-resistance</td>
<td>High-resistance</td>
<td>Low-resistance</td>
</tr>
<tr>
<td>Strokes shift</td>
<td>24nm</td>
<td>100-500nm</td>
<td>&lt; 30nm</td>
</tr>
<tr>
<td>Size and shape</td>
<td>115nm</td>
<td>10-20nm</td>
<td>&lt; 10nm</td>
</tr>
</tbody>
</table>

New technology, cyclic immunofluorescence (CycIF), is a powerful approach to highly multiplexed imaging by building four to six channels at a time [22]. This procedure increasing the multiplicity of cell and tissue staining by cycling the sample involved heat and exposure to acid. CycIF with multiplexed image analysis to deliver highly quantified data, allows for more comprehensive pathological assessment. ER, PR, HER2, and Ki67 were analyzed in a multiplexed assay with similar expression when stained alone, or combined in a multiplexed assay [23]. However, we are worried about some problems to organic fluorescent dyes, including inadequate fluorescent efficiency, low photostability, and autofluorescence interference for precise quantification [24].

**Development of nanoparticle-based IHC:** Quantum dot (QD), a semiconductor nanoparticle, have unique photophysical properties, such as size-tunable symmetric emission bands, superior light absorbance, high fluorescent intensity, and strong photostability [25]. Their unique optical properties have led QD-based nanotechnology to be applied in a wide variety of biomedical applications for cancer diagnosis in combination with cancer biomarkers. Immunofluorescence IHC is thought to be the most common application of QD. Many studies have investigated ER, PR, and HER2 proteins on a single slide correlate closely with the results achieved from traditional IHC, western blot analysis, and fluorescence in situ hybridization [26]. Additionally, they used five QD colors simultaneously on a single clinical tissue specimen to detect five unique markers (ER, mammalian target of rapamycin, PR, epidermal growth factor, and HER2), further demonstrating the molecular profiling potential of these nanoparticles in complex tissue samples [26]. Recently, QD-based double fluorescent imaging technique could help the quantitative study on the co-expressions of Ki67 and HER2 in breast cancer, and Ki67 has a greater negative impact on breast cancer prognosis than HER2 [27]. As quantitative analysis, it was reported the total HER2 load was a good indicator to assess prognosis in breast cancer patients [24]. This parameter identified a distinct subgroup of patients with particularly poor 5-year disease-free survival who were not identified using other methods. Tissue autofluorescence in FFPE samples is comparable to the fluorescent intensity of QD, complicating analysis; quantitative analysis of QD at the single-particle level may be difficult in FFPE samples. However we reported the quantitative sensitivity of immunohistochemistry with QD by using an autofluorescence-subtracted image and single-QD imaging [28]. And Kwon et al developed a new auto-fluorescence
Table 2: Comparison between Nano-patho and other methods for detection of biomarker protein

<table>
<thead>
<tr>
<th>Parameters</th>
<th>IHC</th>
<th>CIFM</th>
<th>QD-IHC</th>
<th>Avi-TMR-IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantification</td>
<td>Medium</td>
<td>Good</td>
<td>Good</td>
<td>Good</td>
</tr>
<tr>
<td>Morphological</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>observation</td>
<td>Good</td>
<td>Good</td>
<td>Good</td>
<td>Good (HE simultaneous stain)</td>
</tr>
<tr>
<td>Multicolor</td>
<td>Poor</td>
<td>Good</td>
<td>Good</td>
<td>no data</td>
</tr>
<tr>
<td>observation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Technical procedure</td>
<td>Eas</td>
<td>Medium</td>
<td>Difficult</td>
<td>Easy</td>
</tr>
</tbody>
</table>

removal method using auto-fluorescence libraries [29]. Although there are some limitations that this technique requires special reagents or instrumentation, and is expensive, QD still has a potential to change the conventional diagnostic system.

Fluorescent dye-containing nanoparticles and avidin-tetramethylrhodamine (Avi-TMR) particles: Fluorescent dye-containing nanoparticles have been shown to have extensive applications in bio-analysis and biodetection due to their high stability and superior luminescence quantum yield [30]. The fluorescence intensity of organic fluorescent material-assembled nanoparticles, termed Avi-TMR (Table 1), is approximately 10-fold greater than that of QDs, and the level of ER expression can be quantified with much greater accuracy compared to DAB-based IHC. These low Stokes shift may lead to a loss of antigen’s detection. But the fluorescent intensity of our Avi-TMR was 10-fold greater than QDs, Avi-TMR IHC produced a significantly higher signal-to-noise ratio on IHC-imaged cancer tissues than previous methods. In addition, the investigation of nuclear morphology by counterstaining with hematoxylin is essential. Hematoxylin staining does not affect Avi-TMR particle staining patterns, indicating that biomarker diagnosis by Avi-TMR particles and nuclear morphology diagnosis using hematoxylin can be performed in identical tissue specimens. A summary of the comparison between Avi-TMR-IHC and common methods for detection of biomarker proteins is shown in Table 2. Some modalities are clinically and experimentally available for detection of biomarker protein at the FFPE sample. Of these technologies, Avi-TMR IHC is particularly appealing due to their very high quantification capabilities (Table 2). This new technology has a potential to achieve the supersensitive quantitative investigation of cancer biomarkers at the single protein level. However, there are several limitations to these studies. In particular, these studies were retrospective, used nonrandomized clinical data sets, and were not validated. Therefore, further studies are required to investigate the applicability of Avi-TMR particles.

Conclusion

The role of fluorescent IHC in bio-imaging and analysis has grown considerably over the last decade, and continued expansion of the applications of this method is expected. Quantitative and objective investigation of cancer biomarkers in FFPE samples is crucial for the development of more accurate diagnostic methods in cancer management. The end purpose of an IHC protocol should be to use IHC not as just a stain, but rather as a precise immunoenassay that is strictly quantifiable at the single protein level. Preclinical nanoparticle methodologies for pathology, as described herein, are expected to have practical benefits for the determination of accurate diagnoses and optimal molecular targeting therapeutic approaches in patients with breast cancer.

Competing interests

We declare that there are no conflicts of interest that could be perceived as affecting the impartiality of the research reported.

Authors’ contributions

All authors participated in the work and made substantial contributions to conception, literature search, manuscript writing, data collection, and data interpretation. KG and MM drafted the article. NO, modified the manuscript. KG carried out the literature search. All authors participated in critical revisions, have granted approval of the final manuscript, and agree to be accountable for all aspects of the work.

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