

The Visualization of Protein-Protein Interactions in Breast Cancer: Deployment Study in Pathological Examination

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The therapeutic strategy is determined by protein expression using immunohistochemistry of estrogen receptor (ER), progesterone receptor, and human epidermal growth factor receptor 2 (HER2) in formalin-fixed paraffin-embedded (FFPE) breast cancer tissues. However, few proteins function independently, and many of them functions due to protein-protein interactions (PPIs) with other proteins. Therefore, it is important to focus on PPIs. This review summarizes the PPIs of ER and HER2 in breast cancer, especially those using a proximity ligation assay that can visualize PPIs in FFPE tissues. In particular, assessing the interaction of CEACAM6 with HER2 may serve as a surrogate marker for the efficacy of trastuzumab in patients with breast cancer. Therefore, in this review, the technique used to detect the interaction of CEACAM6 and HER2 in routinely processed pathological specimens will be applied to the clinical practice of drug selection. We showed the possibility as a novel pathological examination method using PPIs.

Key words: protein-protein interaction, breast cancer, estrogen receptor, human epidermal growth factor receptor 2, proximity ligation assay

I. Introduction

Over 80% of proteins function by forming complexes rather than staying as a single unit [7]. The function of many proteins (signal transduction, transport, and metabolism) depends on structural changes and reactions caused by interactions with other proteins. Therefore, protein-protein interactions (PPIs) are crucial to protein functions, and elucidating PPIs helps in understanding the biological characteristics. It has been reported that aberrant PPIs are implicated in the development of cancer cells. Therefore, clarifying the significance of PPIs is an essen-

tial strategy to treat diseases [28]. Similarly, we have previously reported on PPIs in hormone-dependent cancers [32]. Immunoreactivity with the estrogen receptor α (ER α), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) helps in assessing the prognosis, predictors, and indication of therapeutics in breast cancer [2]. For instance, ER α -positive and/or PR-positive breast cancer patients are treated with endocrine therapy; meanwhile, HER2-positive breast cancer patients are treated with HER2 inhibitor [2]. Endocrine therapy (drugs include tamoxifen and aromatase inhibitors) targets estrogen action, is used in ER and/or PR-positive breast cancer, and has clinical benefit. However, their efficacy is limited by intrinsic and acquired therapeutic resistance [33–35]. HER2 inhibitors (HER2i), such as trastuzumab, enhances the clinical benefit of first-line chemotherapy in patients with HER2-positive breast cancer. Although the effect of trastuzumab in combination with chemotherapy is known, the therapeutic effect of trastuzumab monotherapy is not

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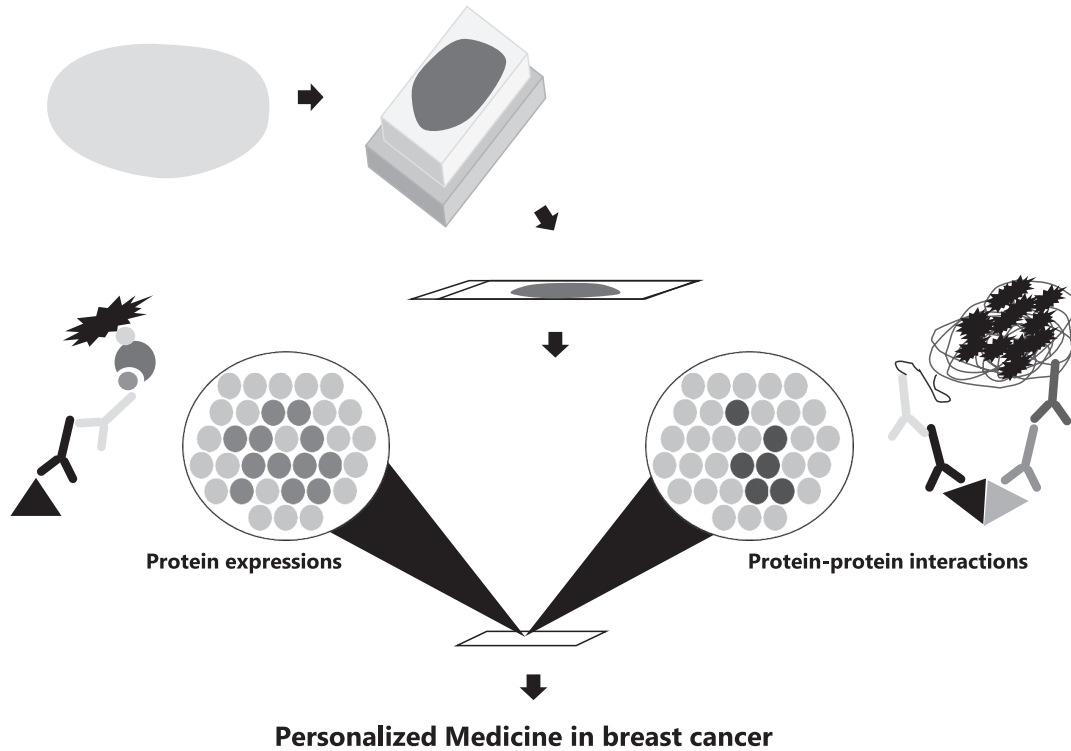


Fig. 1. Application of protein-protein interactions in pathological examination. Diagram showing the implementation of personalized medicine utilizing visualization of protein expression and protein-protein interactions. A specimen is submitted as a surgical resection, a thinly cut sample is mounted in a paraffin block, a thinly cut section is mounted on a glass slide, and then the protein expression and protein-protein interaction analysis was examined.

always clinically sufficient [5, 15, 41, 46]. Therefore, we sought to examine PPIs that contribute to providing appropriate medical care to patients, by providing personalized medicine. Studies using gene analysis are being conducted for the implementation of personalized medicine, but we focused on PPIs using histopathological specimens, that is, protein expression analysis performed by immunohistochemistry using formalin-fixed paraffin-embedded (FFPE) tissues [2]. Therefore, a new pathological examination method can be proposed by PPI analysis using FFPE tissue and immunohistochemistry (Fig. 1). Proximity ligation assay (PLA) analysis was developed to visualize PPIs and can be applied to human tissues [43]. This review summarizes the effects of these biomarkers on PPIs and breast cancer cells, and to use PLA analysis in breast cancer cells.

II. Dimerization of Estrogen Receptors

Approximately 70% of patients with breast cancer expressed the ER [33]. ERs have two isoforms, ER α and ER β , which are well known to form homodimers (α/α , β/β) or heterodimers (α/β) [25]. In ER α -positive breast cancer, cancer cells are promoted by estrogen binding to the ER α . However, ER β inhibited the cell proliferation induced via ER α [29]. Therefore, it is also known that their dimerization has different effects on the intracellular signal transduction mechanism. Estrogen-induced cell proliferation was promoted by the ER α homodimer but inhibited by

the ER β homodimer [29]. ER β inhibited ER α -mediated cell proliferation in co-expression with ER α and ER β , and thus the ER heterodimer inhibited cell proliferation [29]. Therefore, it is necessary to assess the status of ER dimerization in patients with breast cancer and consider the potential effects of estrogen on cancer cell proliferation. By using PLA and structured illumination microscopy (SIM), we have previously reported the presence of ER α homodimers and ER heterodimers in breast cancer cells and tissues [16, 17]. Due to the diffraction limit of light, the resolution of conventional fluorescence microscopes including the laser scanning confocal microscope is 200 nm [47]. Therefore, many proteins may appear to be colocalized, hindering detailed analysis of spatial relationships. Also, the SIM summarized in this study has a resolution of 100 nm, and may be used to evaluate its close proximity, such as PPIs [47]. In both PLA and SIM, the ER α homodimer signal was higher in MCF-7 (ER α -positive) cells than in T-47D cells (ER α -negative) and was not detected in MDA-MB-231 cells (ER α -negative) [16, 17]. In addition, in MCF-7, E2 induced-ER α homodimers increased by minute 15 and 45; in both PLA and N-SIM analysis, the peak of the ER α homodimer occurred at 45 min of E2 treatment [16, 17]. ER heterodimers were detected by E2 treatment at 15 min in MCF-7 cells. The expression of ER α was similar in MCF-7 cells and T-47D cells, and the expression of ER β was higher in T-47D cells than in MCF-7 cells [17]. Therefore, compared to the ER heterodimer, the ER α homodimer

may be predominantly induced by E2 [17]. In T-47D, the ER α homodimer did not increase after 15–90 min of E2 treatment. Therefore, the E2-induced ER β homodimer is detected in breast cancer cells with high ER β expression. The dimerization pattern of ER may depend on the expression profile and the protein expression of the ER isoform in breast cancer cells. Both the ER α homodimer and ER heterodimer were detected using PLA in breast cancer tissues [17]. The ER α homodimer in breast cancer cells was positively associated with the status of ER α and PR, which is an ER target gene considered to be derived from the activation of ER. ER heterodimer in breast cancer cells was also positively associated with the status of ER α , but not associated to both PR and ER β [17]. These results indicated that ER α activity in breast cancer tissues could be evaluated by detecting the ER α homodimer using PLA.

There are several studies on the detection of ER dimers or PPIs with ER using PLA. Majumdar *et al.* found that membrane ER α /ER β 2 heterodimers activated MAPK (Mitogen-activated protein kinase) and AKT pathways in prostate stem and progenitor cells [30]. Jehanno *et al.* showed changes in the interaction between ER α and coactivators or corepressors using PLA when myocardium-related transcription factor A accumulates in the nucleus, and enhances MAPK and AKT activities in MCF-7 cells [21]. Gandhi *et al.* also reported that interaction of p53 with ER α and AMPK was determined by PLA, indicating that ER α -p53 crosstalk could contribute to mTOR pathway [12]. In addition, the visualization of the interaction between ER and PR was seen. Concerning the interactions between ER and PR isoform, PR-B frequency was an independent predictive factor for relapse, whereas PR immunoreactivity was not. Snell *et al.* concluded that ER and PgR-B interactions could be used in predicting patient response to adjuvant aromatase inhibitor therapy [42]. Konan *et al.* showed that ER α -36 interacts constitutively with PR in the nucleus of tumor cells. ER α -36 binds to PgR, and regulates signal transduction, and interferes with its transcriptional activity, progesterone-induced antiproliferative effects, and migratory capacity [23]. Heterogeneous nuclear ribonucleoprotein (hnRNP), is involved in chromatin remodeling, transcription, splicing, and translation processes, and as reported as a binding protein of ER α . It directly interacted with ER α and was involved in the ER-mediated signaling pathway in breast cancer [20]. The interaction between hnRNP and ER α could stabilize ER α and enhance the patient's therapeutic response to endocrine therapy. Furthermore, we reported that Fe65, which is a binding protein of amyloid precursor protein (APP), translocates into the nucleus by phosphorylation of APP and is involved in promoting the cancer cell proliferation and cancer cell migration in breast cancer cells [48]. In addition, Fe65 binds to ER, therefore further investigations are required to clarify effects of proteins that bind to ER on estrogen signaling.

There are various studies on the mechanism of

endocrine therapy resistance, such as ER mutation, the presence of ER β , and crosstalk with growth factor receptors [33–35]. Moreover, the alternative components of the NF κ B signal transduction cascade could directly interact with ER or ER coactivators [38]. The effects of endocrine therapy could be visualized by using PPI analysis in combination with ER, as well as the formation of ER homodimers and heterodimers.

III. Dimerization of HER Family

Human epidermal growth factor receptor 2 (HER2) belongs to the HER family of receptor tyrosine kinases and is overexpressed in approximately 10–34% of breast cancers [37]. Ligand binding and/or receptor overexpression form homodimer or heterodimer, transphosphorylation of the kinase domains, and subsequent activation of downstream signaling pathways, such as the phosphoinositide 3-kinase (PI3K)–AKT and MAPK pathways [10, 31]. The HER family has four members: EGFR (epidermal growth factor receptor), HER2, HER3, and HER4 [9]. Binding of ligands leads to the homodimer and heterodimer formation of the receptor tyrosine kinase [49]. There are several reports on the visualization of HER family dimers. The HER2 homodimer and HER2/HER3 heterodimer has been reported in breast cancer tissues using PLA. Both high levels of HER2 homodimers and HER2/HER3 heterodimers were significantly associated with reduced relapse-free and overall survival [44]. Multiple protein complexes involving EGFR, HER2, and HER3 homodimers and heterodimers have been reported in breast cancer tissues [26]. Barros *et al.* reported that 74%, 66%, and 58% of HER2-positive cases showed heterodimers with EGFR, HER3, and HER4, respectively. HER2 heterodimers were associated with aggressive clinicopathological features and poor outcome [4]. *In situ* PLA also allowed the detection of HER family dimers in non-small cell lung cancer (NSCLC) tissue, and quantitative assessment of EGFR homodimers and heterodimers could contribute to predict the response of patients with NSCLC to EGFR-tyrosine kinase inhibitor treatment [27].

IV. Resistance to HER2 Inhibitors

HER2 inhibitors such as trastuzumab are used therapeutically in HER2-positive breast cancer. The antitumor effects of trastuzumab involve the inhibition of HER2-mediated signaling and activation of antibody-dependent cellular cytotoxicity (ADCC) [45]. PPIs between HER2 and other proteins, except proteins in the HER family, have been reported to affect HER2 signaling and subsequent therapeutic efficacy of HER2 inhibitors. Trastuzumab resistance in breast cancer is through HER2 and CUB domain-containing protein 1 (CDCP1) cooverexpression. This leads to increased transformation ability, cell migration, and tumor formation, enhanced HER2 activation,

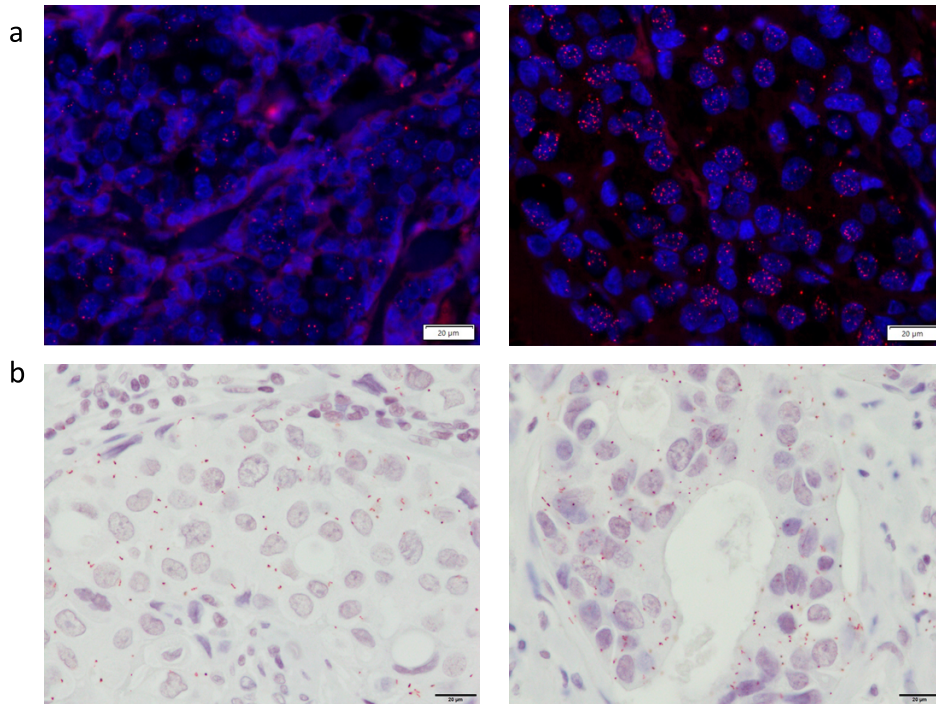


Fig. 2. Detection of PPIs in breast cancer tissues. (a) Detection of ER α homodimers using Texas red in ER α -positive breast cancer tissues. Right shows a high PLA score. Left shows the middle PLA score. (b) Detection of PPIs between HER2 and CEACAM6 using Nova red in both HER2 and CEACAM6 positive breast cancer tissues. Right shows a high PLA score. Left shows a middle PLA score. Bars = 20 μ m.

and downstream signaling [1]. Also, interaction between HER2 and CEACAM6 could be related to trastuzumab sensitivity [18]. CEACAM6 belongs to the CEA family and plays an important role as an intracellular adhesion molecule in many normal tissues including the lung, mammary gland, and colon [6]. In our study, we used HER2i-sensitive BT-474 and resistant MDA-MB-361, which express the CEACAM6 protein [18]. CEACAM6 knockdown by siRNA decreased the sensitivity of BT-474 cells to trastuzumab by both HER2 signaling and ADCC activity, suggesting that CEACAM6 is involved in trastuzumab sensitivity in breast cancer cell [18]. In addition, an interaction between HER2 and CEACAM6 was detected by PLA, immunoprecipitation, and super-resolution imaging analysis in BT-474 cells [18]. CEACAM6 knockdown inhibited HER2 internalization, thus PPIs between HER2 and CEACAM6 contribute to antitumor effects of trastuzumab via HER2 signaling and ADCC activity, by suppressing HER2 internalization [18]. In addition, HER2 and CEACAM6 interactions were detected using PLA in CEACAM6 and HER2 positive breast cancer tissues, and their PLA score was significantly associated with trastuzumab treatment efficacy [18].

Several cell adhesion molecules are involved in HER2 signaling and the subsequent therapeutic effect of HER2 inhibitors. MUC4, a membrane-associated mucin, has been reported to mask HER2 and reduce trastuzumab binding to HER2-positive breast cancer cells. In pancreatic cancer, MUC4 interacted with HER2 and helped promote can-

cer cell growth and metastasis [39]. PPIs between CD44 and HER2 on the cell surface are involved in stimulating CD44-associated HER2 tyrosine kinase activity by hyaluronic acid and promoted ovarian cancer proliferation [3]. These studies could be applied to pathological examinations by visualizing PPIs in human tissues. In addition, previously reported PPIs such as CD44 and HER2 can be analyzed in tissues by using PLA, which may contribute to proper drug selection by PPI analysis.

V. Heterodimerization of CEACAM6 and CEACAM8

Similar to CEACAM6, CEACAM8 also belongs to the CEA family and is involved in cell adhesion, and intracellular and intercellular signaling [6]. The CEA family has also been reported to form homodimers and heterodimers via the N-terminal IgV domain [8, 24, 36]. CEACAM1 and 5 have been reported to form homodimers with strong affinity. CEACAM6 and 8 form homodimers with very weak affinity, however, they form heterodimers with stronger affinity [40]. Heterodimerization of CEACAM6 and CEACAM8 has been reported in neutrophils, which increase cell adhesion to endothelial cells through CD11/CD18 activation [40]. However, the effects of CEACAM6 and CEACAM8 heterodimers in cancer cells are still unclear. Therefore, MCF-7 cells that were stably transfected with CEACAM8 in a previous study demonstrated CEACAM6 and CEACAM8 interaction using PLA

[19]. Coexpression of CEACAM6 and CEACAM8 in MCF-7 cells suppressed the proliferative capacity and transendothelial invasion compared to either CEACAM6 or CEACAM8 expression [19]. Furthermore, immunohistochemical analysis using breast cancer tissues also indicate that CEACAM6 and CEACAM8 double-positive carcinoma cells were of a low histological grade and stage than single-positive or double-negative cells [19]. Therefore, CEACAM6 or CEACAM8 alone contributed to promote cell proliferation and transendothelial invasion in breast cancer cells, but PPIs between CEACAM6 and CEACAM8 in cancer cells could inhibit the adhesion to vascular endothelial cells and subsequent collapse of endothelial junctions. Some studies showed the involvement of adhesion molecules in specific organs in cancer metastasis. High expression of activated ALCAM has been reported in the skin metastases of breast cancer cells [14]. Increased expression of E-selectin has also been reported in inflammation-related lung metastases of mouse breast cancer [22]. CEACAM6 expression in our study was higher in bone than in lung metastases [19]. CEACAM6 expression of pancreatic adenocarcinoma cells interact with $\alpha\beta 3$ integrin, and subsequent increased adhesion of ECM components, and can result in cell invasion and metastasis [11]. Therefore, we believe the PPIs between CEACAM6 and CEACAM8 may differ in the metastatic microenvironment of the bone and lung. In addition, some studies showed that the CEA family interacted with other proteins. CEASLex interactions have been reported as aggressive tumor features in gastric cancer, and detection of their PPIs using PLA was useful as a biomarker for the prognosis of patients with gastric cancer in theranostic applications [13]. The CEA family dimer pattern and/or PPIs with other proteins, and its function are not well known and therefore further studies are needed. On the other hand, there was a difference in the affinity to form homodimer and/or heterodimer in the CEA family [40]. The PLA method can also be used to perform a multiplex proximity ligation assay to simultaneously visualize multiple protein complexes *in situ* [26]. Therefore, in tumors with multiple CEA family proteins, the simultaneous detection of multiple PPIs may reveal differences in dimer formation affinity and the function of dimer patterns.

VI. Conclusion

Assessment of CEACAM6 and HER2 interaction may be a surrogate marker for the efficacy of trastuzumab in patients with breast cancer. Therefore, this review showed that the techniques used to detect CEACAM6 and HER2 interactions in routinely processed pathological specimens, in addition to assessing the HER2 status, are useful methods to select drugs in clinical practice. PPIs with the ER and CEA family were also successfully detected in both breast cancer cells and human breast cancer tissues, but further studies are needed to clarify the possibility of appli-

cation of visualization of PPIs to pathological examination. PLA can be analyzed using FFPE tissues and immunohistochemistry, and is suitable for examination using a relatively large number of cases on pathological diagnosis. In addition, PPI analysis by PLA can be observed with an optical microscope using nova red and Texas red for a fluorescence microscope. This can be performed even in a facility without a fluorescence microscope (Fig. 2). We showed the possibility of using PPIs as a novel pathological examination method. PPI analysis can lead to proper use during endocrine or anti-HER2 therapy.

VII. Conflicts of Interest

All authors declare that they have no conflict of interest.

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