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The Human Epidermal Growth Factor Receptor 2 (HER2) as a Prognostic and Predictive Biomarker: Molecular Insights into HER2 Activation and Diagnostic Implications

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Abstract

The human epidermal growth factor receptor 2 (HER2) is a transmembrane tyrosine kinase receptor protein. *HER2* gene amplification and receptor overexpression, which occur in 15–20% of breast cancer patients, are important markers for poor prognosis. Moreover, HER2-positive status is considered a predictive marker of response to HER2 inhibitors including trastuzumab and lapatinib. Therefore, reliable HER2 determination is essential to determine the eligibility of breast cancer patients to targeted anti-HER2 therapies. In this chapter, we aim to illustrate important aspects of the HER2 receptor as well as the molecular consequences of its aberrant constitutive activation in breast cancer. In addition, we will present the methods that can be used for the evaluation of HER2 status at different levels (protein, RNA, and DNA level) in clinical practice.

Keywords: breast neoplasm, oncogene, tyrosine kinase receptor, molecular oncology, HER2 status, HER2 inhibitors

1. Introduction

Breast cancer is the most frequently diagnosed cancer among women worldwide, affecting over 1.5 million women each year. In 2015, it is estimated that worldwide 500,000 women have died from this malignancy, which represents 15% of all cancer-related deaths among women [1].

It is now well recognized that breast cancer comprises a heterogeneous group of diseases in term of differentiation and proliferation, prognosis and treatment. Over the past decades, microarray-based gene expression studies have allowed the identification of breast cancer intrinsic subtypes [2–4]. One of these subtypes is the so-called human epidermal growth factor receptor 2 (HER2)-enriched subtype. HER2 is a transmembrane tyrosine kinase receptor [5]. This protein is encoded by the *HER2* gene, which is located on the long arm of chromosome 17 (17q12–21.32) [6]. The HER2-enriched subtype is characterized by high expression of HER2 and other genes of the 17q amplicon, including growth factor receptor bound protein 7 (GRB7), and low to intermediate expression of luminal genes such as Estrogen Receptor 1 (ESR1) and Progesterone Receptor (PGR) [7]. Clinically, HER2-positive breast cancer occurs in 15–20% of breast cancer patients and is characterized by the overexpression of the HER2 receptor and/or *HER2* gene amplification [8]. HER2-positive breast cancer patients have a particular worse prognosis. Importantly, HER2-positive breast cancer patients are eligible to receive targeted treatment with trastuzumab, a monoclonal antibody specifically directed against the HER2 receptor [9]. Trastuzumab treatment, in combination with chemotherapy, improves the outcome of early [10, 11] and metastatic [12, 13] HER2-positive breast cancer patients. The US Food and Drug Administration (FDA) approved trastuzumab for the treatment of metastatic HER2-positive breast cancer patients in 1998 and for the treatment of early HER2-positive breast cancer patients in 2006. Lapatinib is a small-molecule inhibitor of the intracellular tyrosine kinase domain of both HER2 and EGFR receptors [14]. Lapatinib has received FDA approval in 2007 as combination therapy with capecitabine for the treatment of patients with HER2-positive advanced breast cancer patients who had progressed on trastuzumab-based regimens [15]. Although anti-HER2 agents are generally well tolerated, trastuzumab administration has been associated with cardiac side effects, especially when used in combination with anthracyclines [16].

HER2 plays a significant role in breast cancer pathogenesis. It is therefore essential to understand the biology of this receptor in order to better treat HER2-positive breast cancer patients. Evaluation of HER2 status in breast cancer specimens raises several technical considerations. In the last decades, several methods have been developed for HER2 assessment. In this article, we will review important aspects of the HER2 biology and its relevance in breast cancer and present the techniques that are used in clinical practice for the determination of HER2 status in breast cancer specimens.

2. HER2 biology and methods of assessment of HER2 status

2.1. HER2 receptor

The HER2 receptor is a 185 kDa transmembrane protein that is encoded by the *HER2* (also known as *erb-b2 receptor tyrosine kinase 2* [*ERBB2*]) gene, which is located on the long arm of chromosome 17 (17q12–21.32) [6]. HER2 is normally expressed on cell membranes of epithelial cells of several organs like the breast and the skin, as well as gastrointestinal, respiratory, reproductive, and urinary tract [17]. In normal breast epithelial cells, HER2 is expressed at low levels (two copies of the *HER2* gene and up to 20,000 HER2 receptors) [18], whereas in HER2-positive breast cancer cells, there is an increase in the number of *HER2* gene copies (up to 25–50, termed gene amplification) and HER2 receptors (up to 40 to 100 fold increase,

termed protein overexpression), resulting in up to 2 million receptors expressed at the tumor cell surface [19]. Besides breast cancer, HER2 overexpression has also been reported in other types of tumors, including stomach, ovary, colon, bladder, lung, uterine cervix, head and neck, and esophageal cancer as well as uterine serous endometrial carcinoma [20].

2.1.1. HER2 structure and function

HER2 belongs to the epidermal growth factor receptor (EGFR) family. This family is composed of four HER receptors: human epidermal growth factor receptor 1 (HER1) (also termed EGFR), HER2, human epidermal growth factor receptor 3 (HER3), and human epidermal growth factor receptor 4 (HER4) [5].

HER family members are transmembrane receptor tyrosine kinases. Tyrosine kinases are enzymes that carry out tyrosine phosphorylation, namely the transfer of the γ phosphate of adenosine triphosphate (ATP) to tyrosine residues on protein substrate [21].

HER receptors share a similar structure. They are composed of an extracellular domain (ECD), a transmembrane segment and an intracellular region [22]. The ECD domain is divided into four parts: domains I and III, which play a role in ligand binding, and domains II and IV, which contain several cysteine residues that are important for disulfide bond formation [23]. The transmembrane segment is composed of 19–25 amino acid residues. The intracellular region is composed of a juxtamembrane segment, a functional protein kinase domain (with the exception of HER3 that lacks tyrosine kinase activity [24] and must partner with another family member to be activated [25]), and a C-terminal tail containing multiple phosphorylation sites required for propagation of downstream signaling [23]. The catalytic domain contains the ATP binding pocket, a conserved site essential to ATP binding [26].

HER receptors are activated by both homo- and heterodimerization, generally induced by ligand binding [27]. This suggests that HER receptor family has evolved to provide a high degree of signal diversity [28]. The cellular outcome produced by HER receptors activation depends on the signaling pathways that are induced, as well as their magnitude and duration, which are influenced by the composition of the dimer and the identity of the ligand [28].

Several growth factor ligands interact with the HER receptors [29]. HER1 receptor is activated by six ligands: epidermal growth factor (EGF), epigen (EPG), transforming growth factor α (TGF α), amphiregulin, heparin-binding EGF-like growth factor, betacellulin and epiregulin. HER3 and HER4 receptors bind neuregulins (neuregulin-1, neuregulin-2, neuregulin-3, and neuregulin-4). HER2 is a co-receptor for many ligands and is often transactivated by EGF-like ligands, inducing the formation of HER1-HER2 heterodimers. Neuregulins induces the formation of HER2-HER3 and HER2-HER4 heterodimers [29]. However, no known ligand can promote HER2 homodimer formation, implying that no ligand can bind directly to HER2 [30].

The structural basis for receptor dimerization has been elucidated in recent years through crystallographic studies [31, 32]. Dimerization is mediated by the dimerization arm, a region of the extracellular region of HER receptors. While in its inactivated state the dimerization arm of EGFR, HER3 and HER4 is hidden, ligand binding induces a receptor conformational change leading to exposure of the dimerization arm [31]. In contrast to the other three HER receptors, the dimerization arm of the HER2 receptor is permanently partially exposed, thus permitting its dimerization even if the HER2 receptor lacks ligand-binding activity [32].

Interaction between the dimerization arms of two HER receptors promotes the formation of a stable receptor dimer in which the kinase regions of both receptors are closed enough to permit transphosphorylation of tyrosine residues, i.e. the transfer of a phosphate group by a protein kinase to a tyrosine residue in a different kinase molecule [33, 34]. The first member of the dimer mediates the phosphorylation of the second, and the second dimer mediates the phosphorylation of the first [23].

The phosphorylation of specific tyrosine residues following HER receptor activation and the subsequent recruitment and activation of downstream signaling proteins leads to activation of downstream signaling pathways promoting cell proliferation, survival, migration, adhesion, angiogenesis and differentiation [35]. The Phosphatidylinositol 3'-kinase (PI3K)-Akt pathway and the Ras/Raf/MEK/ERK pathway (also known as extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway) are the two most important and most extensively studied downstream signaling pathways that are activated by the HER receptors [5, 36]. These downstream signaling cascades control cell cycle, cell growth and survival, apoptosis, metabolism and angiogenesis [37, 38]. Signaling from HER receptors is then terminated through the internalization of the activated receptors from the cell surface by endocytosis. Internalized receptors are then either recycled back to the plasma membrane (HER2, HER3, HER4) or degraded in lysosomes (HER1) [39, 40].

HER heterodimers produce more potent signal transduction than homodimers. This can be explained by the fact that heterodimerization provides additional phosphotyrosine residues necessary for the recruitment of effector proteins [28]. Heterodimerization follows a strict hierarchical principle with HER2 representing the preferred dimerization and signaling partner for all other members of the HER family [41]. HER2 seems to function mainly as a co-receptor, increasing the affinity of ligand binding to dimerized receptor complexes [42, 43]. HER2 has the strongest catalytic kinase activity [41] and HER2-containing heterodimers produce intracellular signals that are significantly stronger than signals generated from other HER heterodimers [44]. The HER2-HER3 heterodimer in particular exhibits extremely potent mitogenic activity through the stimulation of the PI3K/Akt pathway, a master regulator of cell growth and survival [45]. Furthermore, HER2 containing heterodimers have a slow rate of receptor internalization, which results in prolonged stimulation of downstream signaling pathways [28]. HER2 can also be activated by complexing with other membrane receptors, such as Insulin-like growth factor I receptor (IGF-1R) [46].

2.1.2. Consequences of constitutive HER2 receptor activation

Whereas in normal cells the activity of tyrosine kinases is a tightly controlled mechanism, in cancer cells, alterations in tyrosine kinases—overexpression of receptor tyrosine kinase proteins, amplification or mutation in the corresponding gene, abnormal stimulation by auto-crine growth factors loop or delayed degradation of activated receptor tyrosine kinase—lead to constitutive kinase activation and therefore to aberrant cellular growth and proliferation [34, 47]. Constitutive activation of HER1, HER2, HER3, IGF-1R, Fibroblast growth factor receptor (FGFR), c-Met, Insulin Receptor (IR), Vascular Endothelial Growth Factor Receptor (VEGFR), Jak kinases and Src have been associated with human cancer [34, 48–52].

Several ways of aberrant activation of HER receptors have been described, including ligand binding, molecular structural alterations, lack of the phosphatase activity, or overexpression of the HER receptor [53].

In HER2-positive tumors, receptor overexpression has been identified as the mechanism of HER2 activation. The increased amount of cell surface HER2 receptors associated with HER2 overexpression leads to increased receptor-receptor interactions, provoking a sustained tyrosine phosphorylation of the kinase domain and therefore constant activation of the signaling pathways. HER2 overexpression also enhances HER2 heterodimerization with HER1 and HER3 [54] resulting in an increased activation of the downstream signaling pathways. It has also been shown that HER2 overexpression leads to enhanced HER1 membrane expression and HER1 signaling activity through interference with the endocytic regulation of HER1 [54–56]. While HER1 undergoes endocytic degradation after ligand-mediated activation and homodimerization, HER1-HER2 heterodimers evade endocytic degradation in favor of the recycling pathway [57, 58], resulting in increased HER1 membrane expression and activity [55, 56, 59].

It has also been reported that HER2 overexpression enhances cell proliferation through the rapid degradation of the cyclin-dependent kinase (Cdk) inhibitor p27 and the upregulation of factors that promote cell cycle progression, including Cdk6 and cyclins D1 and E [60].

Several methods have been developed for the assessment of HER2 status in breast cancer specimens, at the protein level, DNA level, and RNA level. Here below, we present some of the existing techniques that are used for the HER2 determination in clinical practice.

2.2. Methods for the evaluation of HER2 status in breast cancer specimens

2.2.1. HER2 status evaluation at the protein level

2.2.1.1. Immunohistochemistry (IHC)

IHC allows the evaluation of the HER2 protein expression in formalin-fixed, paraffin-embedded (FFPE) tissues using specific antibodies directed against the HER2 receptor protein [61]. HER2 receptor is then visualized with the chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB) resulting in a brownish membranous staining. Several commercially available diagnostic tests for the determination of HER2 expression have been approved by the FDA: the HercepTest™ kit (DAKO, Glostrup, Denmark), the InSite™ HER2/neu kit (clone CB11; BioGenex Laboratories, San Ramon, CA), the Pathway™ kit (clone 4B5; Ventana Medical Systems, Tucson, AZ), and the Bond Oracle HER2 IHC System (Leica Biosystems, Newcastle, UK).

By this method, it is possible to estimate the number of cells showing membranous staining in the tissue section as well as the intensity of the staining [62]. Membranous staining in the invasive component of specimen is scored on a semi-quantitative scale. According to the American Society of Clinical oncology (ASCO) and the College of American Pathologists (CAP) recommendations for HER2 testing in breast cancer published in 2013, HER2 expression is scored as 0 (no staining or weak/incomplete membrane staining in ≤10% of tumor cells), 1+ (weak, incomplete membrane staining in >10% of tumor cells), 2+ (strong, complete membrane

staining in $\leq 10\%$ of tumor cells or weak/moderate and/or incomplete membrane staining in $>10\%$ of tumors cells) or 3+ (strong, complete, homogeneous membrane staining in $>10\%$ of tumor cells) [61]. In clinical practice, HER2 immunohistochemical status is evaluated as negative if the immunohistochemical score is 0 or 1+, equivocal if the score is 2+, and positive if the score is 3+. Patients with a positive HER2 status at the IHC are eligible for targeted therapy with HER2 inhibitors. The IHC 2+ category is considered borderline and confirmatory testing using an alternative assay (fluorescence *in situ* hybridization (FISH) or other *in situ* hybridization (ISH) methods, see Section 2.2.2) is required for final determination.

IHC is an easy and relatively inexpensive method [63]. However, this technique can be affected by numerous factors, including warm/cold ischemic time [64], delay and duration of fixation [65], and antibody used [66, 67]. Moreover, since the interpretation of results is based on semiquantitative scoring, this technique is prone to interobserver variability and therefore to substantial discrepancies in the IHC results, particularly for cases scoring 2+ [68].

2.2.1.2. Enzyme-linked immunosorbent assay (ELISA)

As mentioned before, HER2 receptor is composed of an extracellular domain (ECD), a transmembrane domain, and an intracellular domain with tyrosine kinase activity. The HER2 ECD can be cleaved from the HER2 full-length receptor through matrix metalloproteases and released into the serum [69]. HER2 ECD levels present in serum can be measured using an enzyme-linked immunosorbent assay (ELISA). HER2 ECD is detected using two antibodies that recognize two specific epitopes of the antigen. Several commercially available ELISA assays received FDA approval: the automated ELISA assay Immuno-1 (Siemens Healthcare Diagnostics, Tarrytown, NY), the manual ELISA assay (Siemens Healthcare Diagnostics) in 2000, and the automated ELISA assay ADVIA Centaur (Siemens Healthcare Diagnostics) in 2003 [70].

Although some studies suggest that HER2 ECD levels measured in patient's serum could be used as a biomarker for the monitoring of the disease course and the response of the patient to therapy, the clinical use of the ELISA assay for the evaluation of the HER2 ECD has not yet been widely implemented [71, 72]. This is mainly due to the fact that studies that analyzed the association between HER2 ECD levels and prognostic and predictive factors in breast cancer patients reported conflicting results, depending on which cutoff value was considered or which assay was used [71].

ELISA is an easy and fast method. In addition, given that HER2 ECD can be measured directly in serum, ELISA can be used to monitor the dynamic changes of HER2 status following treatment or over the course of the disease progression [71]. Results obtained by ELISA, however, might not be reliable if the serum samples are from patients under treatment, as trastuzumab present in the patient's serum might compete with the two antibodies used in the assay.

2.2.2. HER2 status evaluation at the DNA level

2.2.2.1. Fluorescence *in situ* hybridization (FISH)

The FISH technique is a cytogenetic technique that uses fluorescent probes to target specific DNA sequences in FFPE tissue samples [73]. FISH is effectuated either as a single-color assay (HER2 probe only) to evaluate HER2 gene copies per nucleus or as a dual-color assay using

differentially labeled HER2 and chromosome 17 centromere (chromosome enumeration probe 17, CEP17) probes simultaneously. The dual-color assay allows the determination of the HER2/CEP17 ratio [74]. The HER2/CEP17 ratio is often regarded as a better reflection of the *HER2* amplification status, as the latter may be influenced by abnormal chromosome 17 copy number (mainly polysomy) [75].

The *HER2* gene locus on chromosome 17 is recognized by the HER2 probe, which is labeled with a fluorophore (orange as example). The α satellite DNA sequence located at the centromeric region of chromosome 17 is recognized by a fluorophore-labeled chromosome 17 centromere probe (green as example). Nuclei are then counterstained with 4,6'-diamino-2-phenylindole (DAPI). Fluorescent hybridization signals can be visualized using a fluorescence microscope equipped with appropriate filters (for example Spectrum Orange for locus-specific probe HER2, Spectrum Green for centromeric probe 17, and the UV filter for the DAPI nuclear counterstain) [76].

Three FISH assay kits have been approved by the FDA for the determination of the *HER2* gene amplification in breast cancer specimens: the single-probe INFORM HER2 FISH DNA kit (Ventana Medical Systems), the dual-probe PathVysion HER-2 DNA probe kit (Abbott Molecular, Des Plaines, IL), and the dual-probe HER2 FISH PharmDx kit (DAKO).

According to the 2013 ASCO/CAP guidelines, a case is evaluated as amplified when the mean *HER2* gene copy number is ≥ 6 signals/nucleus or HER2/CEP17 ratio is ≥ 2.0 , else as equivocal if mean *HER2* gene copy number is ≥ 4 and < 6 signals/nucleus, and else as non-amplified when the mean *HER2* gene copy number is < 4 signals/nucleus. In order to adequately evaluate *HER2* status, a minimum of 20 tumor cell nuclei are counted in at least two invasive tumor areas. For equivocal FISH specimens, results are confirmed by counting 20 additional cells [61]. Moreover, the equivocal category requires reflex testing with the alternative assay (IHC) on the same specimen for final determination. Reflex testing can also be performed using IHC or ISH methods on an alternative specimen. If specimen is evaluated as equivocal, even after reflex testing, the oncologist may consider targeted treatment.

Although still matter of debate, several researchers consider FISH as being more accurate and reliable than IHC in the assessment of *HER2* status in breast cancer specimens [77–80]. In addition, given that DNA is more stable than protein, preanalytical factors have less impact on assay results compared with IHC [81]. Although the FISH technique yields results that are considered more objective and quantitative than immunohistochemical scoring [73, 82], this method is nine times more time-consuming [83] and three times more expensive compared with IHC [84]. In addition, costly equipment is required for signal detection [67]. The FISH assay can be interpreted only by well-trained personnel, as distinguishing invasive breast cancer from breast carcinoma *in situ* under fluorescence is arduous [85].

Moreover, fluorescence signal counting is time consuming. To overcome this limitation, image analysis software for the automated assessment of fluorescence signals has been developed. Several investigators have reported an excellent concordance between HER2/CEP17 ratios calculated through manual counting and those obtained with automated image analysis system [86–88]. Some image analysis systems has been approved by the FDA for the automated determination of *HER2* gene amplification: the Metafer (MetaSystems, Altlussheim, Germany) and the Ariol HER2/neu FISH (Applied Imaging, San Jose, CA). Furthermore, this software allows the storing of captured images [86].

2.2.2.2. *Bright-field in situ hybridization (ISH) methods*

Given that FISH technology have some limitations, alternative ISH methods have been developed for the assessment of *HER2* gene amplification in breast cancer specimens. Similar to FISH, these methods allow the quantification of *HER2* gene copy number within tumor cell nuclei in FFPE tissues using a DNA probe that specifically recognizes specific DNA sequences. However, whereas the FISH assay is performed with DNA probes that are coupled to a fluorescent detection system, these alternative ISH methods are performed with probes that are coupled to chromogenic (chromogenic ISH [CISH]), or silver detection system (silver-enhanced ISH [ISH]), or a combination of CISH and SISH (bright-field double ISH [BDISH]) [89]. Similar to FISH, ISH methods are performed either as single-color assay or as a dual-color assay.

Since visualization is achieved using other reactions than fluorescence-labeled probe, signals can be evaluated using a standard bright-field microscope, allowing the simultaneous analysis of *HER2* gene amplification and morphologic features of tissues. Moreover, contrary to fluorescent signals that fade over time, bright-field ISH signals are permanent [90]. Here after, we will briefly describe the bright-field ISH methods that are used in clinics.

2.2.2.3. *Chromogenic in situ hybridization (CISH)*

CISH allows the visualization of target genes in breast cancer tissue sections through peroxidase enzyme-labeled probes [90]. The single-color CISH assay (SPOT-Light *HER2* CISH kit; Life Technologies, Carlsbad, CA), and the dual-color CISH assay (*HER2* CISH PharmDx kit; Dako) received FDA approval in 2008 and 2011, respectively [61].

With the single-color CISH assay, only the absolute *HER2* gene copy number is evaluated. The hybridized *HER2* probe is visualized by DAB as chromogen. *HER2* gene copies are recognizable as brown chromogenic reaction product signals within nuclei. Slides are then counterstained with hematoxylin [82, 91, 92]. *HER2* signals are recognizable either as large brownish signal clusters or as numerous individual brownish small signals [92]. Cases with low-level amplification show six to 10 signals per nucleus in more than 50% of breast cancer cells, whereas high-level amplification cases are characterized by a mean *HER2* gene copy number of more than 10 or by large gene copy clusters in more than 50% of breast cancer cell nuclei [92, 93].

The dual-color CISH assay allows the simultaneous visualization of the *HER2* and CEP17 probes on the same slide [94]. *HER2* probes are visualized using a chromogen (green as example), whereas CEP17 probes are visualized using another chromogen (red as example). Slides are then counterstained with hematoxylin. Results obtained by dual-color CISH are reported as dual-color FISH [61].

The CISH assay is twice cheaper [72] and 1.2 times faster [82] comparatively to FISH. Furthermore, since the CISH assay allows an easier identification of the invasive component compared with FISH, evaluation of CISH signals is less time-consuming than FISH [82, 94]. In addition, tumor heterogeneity is promptly recognizable, even at low magnification [95]. Moreover, the dual-color assay can be performed on an automated slide stainer, improving the reproducibility of the assay [96]. However, the assessment of *HER2* gene copy number can be arduous in tumor regions showing high-level amplification, since overlapping dots lead to formation

of signal clusters that are difficult to evaluate [94]. In addition, technical problems, including under- or overfixation, over- or underdigestion of tissue samples can lead to inaccurate results or loss of signals [91, 93].

2.2.2.4. Silver-enhanced *in situ* hybridization (SISH)

SISH is an automated enzyme metallography assay, in which an enzyme reaction is used to selectively deposit metallic silver from solution at the reaction site to produce a black staining [97]. All steps of the assay are performed on the Ventana BenchMark XT automated slide stainer [98, 99]. HER2 and chromosome 17 analysis is performed on sequential slides [98, 99]. As previously mentioned, HER2 and CEP17 probes are visualized through the process of enzyme metallography. During the process, silver precipitation is deposited in the nucleus, and HER2 or CEP17 signals are visualized as black dots within cell nuclei [99]. Similar to the FISH assay, *HER2* gene amplification status assessed by SISH is reported as a HER2/CEP17 ratio, according to the ASCO/CAP guidelines [61].

Given that the SISH assay is fully automated, this technique is six times faster to perform than the FISH assay [99]. In addition, black SISH signals are easier to evaluate compared with other bright-field ISH techniques [100, 101]. However, to correct for chromosome 17 aneuploidy, the hybridization of a further section is required for separate assessment of CEP17 copy number [100].

2.2.2.5. Bright-field double ISH (BDISH)

Bright-field double ISH (BDISH) or dual-color *in situ* hybridization (dual ISH) is a fully automated bright-field ISH assay for the simultaneous determination of HER2 and CEP17 signals on the same FFPE breast cancer tissue sections [100]. This assay combines the visualization of *HER2* gene copies through the deposition of metallic silver particles, similar to the monochrome SISH procedure, with the detection of CEP17 copies with a red chromogen, similar to the CISH assay [102]. HER2 signals are visualized as discrete black spots and the CEP17 signals as red spots in the nuclei. Slides are then counterstained with hematoxylin [100]. *HER2* gene amplification status assessed by BDISH is reported as a HER2/CEP17 ratio, according to the ASCO/CAP guidelines.

This technique is very pertinent especially for cases displaying chromosome 17 aneuploidy or intratumoral heterogeneity, as it allows the simultaneous visualization of both HER2 and CEP17 probes on the same slide [100]. Furthermore, as the HER2 signals and CEP17 signals differ in color and size (HER2 black spots are smaller than CEP17 red spots), both signals can be distinguished from each other, even though they colocalize within cell nuclei [100]. Moreover, since this assay is completely automated, results are available within 6 h, in addition of being more reproducible, as risk of human errors are diminished [101]. The BDISH assay presents the same disadvantages as CISH and SISH.

2.2.2.6. Instant-quality FISH (IQFISH) and automated HER2 FISH

Recently, new FISH assays have been developed for the evaluation of *HER2* gene amplification in breast cancer specimens, including instant-quality FISH (IQFISH), which received

FDA approval, and automated *HER2* FISH. In analogy to conventional FISH, these new assays allow the quantitative determination of *HER2* gene amplification. The IQFISH assay is performed in the same way as manual FISH, with the exception of the hybridization buffer (IQFISH buffer), which considerably reduces the time required for the hybridization step (16 times faster) and therefore the total assay time [103, 104]. Moreover, while hybridization buffer provided in conventional FISH assay contain the toxic formamide, the IQFISH buffer is nontoxic [103]. Compared to conventional FISH, automated FISH is less expensive, since the full automation of the assay requires less human intervention [105]. Furthermore, automated FISH enables faster processing of samples and recording [105].

2.2.3. *HER2* status evaluation at the RNA level

2.2.3.1. Polymerase chain reaction (PCR)-based assays

Polymerase chain reaction (PCR) is a technique used for the detection of DNA samples through the exponential amplification of target DNA sequences.

Reverse transcription PCR (RT-PCR) assay allows the quantification of mRNA and can be used for the evaluation of *HER2* expression in breast cancer specimens in both FFPE and frozen tissues [106, 107]. Extracted mRNA is at first reverse transcribed into complementary DNA (cDNA). cDNA is then measured by quantitative PCR (qPCR). The relative quantitation of *HER2* gene expression is evaluated comparing the target gene expression with that of housekeeping genes. The relative *HER2* gene expression measured in samples is then normalized to a calibrator obtained by mixing RNA from several normal breast tissue specimens. Of note, the Oncotype Dx (Genomic Health, Redwood City, CA) assay is a test based on RT-PCR technology and is used to analyze the expression of 21 genes involved in breast cancer biology, such as *HER2*, ER, and PR. This assay is used to predict the likelihood of breast cancer recurrence in patients with early-stage, node-negative, ER-positive breast cancer [106].

RT-PCR has a large dynamic range, in addition of being a quantitative method. PCR results, however, are often associated with false-negative results due to dilution of amplified tumor cells with surrounding nonamplified stromal cells [108, 109]. In addition, the evaluation of *HER2* status at the mRNA level by RT-PCR using FFPE tissues can be problematic, as mRNA integrity can be damaged by several factors, including tissue fixation and storage time [110].

3. Conclusion(s)

HER2 is a prognostic marker in breast cancer. *HER2* overexpression and *HER2* gene amplification, which occur in 15–20% of breast cancer patients, cause aberrant constitutive activation of the signaling pathway. This leads to uncontrolled and unregulated cell growth and correlates with poor outcome of *HER2*-positive breast cancer patients.

In addition, *HER2*-positive status is considered a predictive marker of response to *HER2*-targeted drugs, including trastuzumab and lapatinib [111]. Considering the clinical and economic implications of targeted anti-*HER2* treatments, reliable *HER2* test results are essential.

False negative results would deny the patients access to the potential benefits of trastuzumab, whereas false positive results would expose patients to the potential cardiotoxic side effects of this expensive agent without experiencing any therapeutic advantages [89].

Although several techniques have obtained FDA approval for the HER2 assessment in breast cancer specimens, the ASCO/CAP guidelines recommend performing IHC or ISH methods to determine HER2 status in breast cancer. The optimal method for evaluating HER2 status in breast cancer specimens, however, is still matter of debate, since each method is characterized by its own advantages and disadvantages. Therefore, emphasis must be put on standardization of procedures and quality control assessment of already existing methods. Also, development of new accurate assays should be promoted. Moreover, large clinical trials are needed to identify the technique that most reliably predicts a positive response to HER2 inhibitors.

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Conflict of interest

The authors have no conflicts of interests to declare.

Notes/thanks/other declarations

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Acronyms and abbreviations

HER2	Human epidermal growth factor receptor 2
GRB7	Growth factor receptor bound protein 7
ESR1	Estrogen Receptor 1
PGR	Progesterone Receptor
FDA	Food and Drug Administration
EGFR	Epidermal growth factor receptor

IHC	Immunohistochemistry
FISH	Fluorescence <i>in situ</i> hybridization
ERBB2	erb-b2 receptor tyrosine kinase 2
HER3	Human epidermal growth factor receptor 3
HER4	Human epidermal growth factor receptor 4
ATP	Adenosine triphosphate
ECD	extracellular domain
EGF	Epidermal growth factor
EPG	Epigen
TGF α	Transforming growth factor α
PI3K	Phosphatidylinositol 3'-kinase
ERK	Extracellular signal-regulated kinase
MAPK	Mitogen-activated protein kinase
FGFR	Fibroblast growth factor receptor
IR	Insulin Receptor
VEGFR	Vascular Endothelial Growth Factor Receptor
Cdk	Cyclin-dependent kinase
FFPE	Formalin-fixed, paraffin-embedded
DAB	3,3'-diaminobenzidine tetrahydrochloride
ASCO	American Society of Clinical Oncology
CAP	College of American Pathologists
ELISA	Enzyme-linked immunosorbent assay
CEP17	Chromosome enumeration probe 17
DAPI	4,6'-diamino-2-phenylindole
ISH	<i>in situ</i> hybridization
CISH	Chromogenic <i>in situ</i> hybridization
SISH	Silver-enhanced <i>in situ</i> hybridization
BDISH	Bright-field double ISH
PCR	polymerase chain reaction

RT-PCR	Reverse transcription PCR
cDNA	Complementary DNA
qPCR	Quantitative PCR

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References

- [1] WHO. 2017. Available from: <http://www.who.int/cancer/prevention/diagnosis-screening/breast-cancer/en/> [Accessed: 2017-03-23]
- [2] Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. *Nature*. 2000;**406**(6797):747-752
- [3] Sorlie T. Molecular classification of breast tumors: Toward improved diagnostics and treatments. *Methods in Molecular Biology*. 2007;**360**:91-114
- [4] Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proceedings of the National Academy of Sciences of the United States of America*. 2003;**100**(14):8418-8423
- [5] Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nature Reviews. Molecular Cell Biology*. 2001;**2**(2):127-137
- [6] Popescu NC, King CR, Kraus MH. Localization of the human erbB-2 gene on normal and rearranged chromosomes 17 to bands q12-21.32. *Genomics*. 1989;**4**(3):362-366
- [7] Prat A, Pascual T, Adamo B. Intrinsic molecular subtypes of HER2+ breast cancer. *Oncotarget*. 2017;**8**(43):73362-73363

- [8] Soerjomataram I, Louwman MW, Ribot JG, Roukema JA, Coebergh JW. An overview of prognostic factors for long-term survivors of breast cancer. *Breast Cancer Research and Treatment*. 2008;**107**(3):309-330
- [9] Yersal O, Barutca S. Biological subtypes of breast cancer: Prognostic and therapeutic implications. *World Journal of Clinical Oncology*. 2014;**5**(3):412-424
- [10] Gianni L, Dafni U, Gelber RD, Azambuja E, Muehlbauer S, Goldhirsch A, et al. Treatment with trastuzumab for 1 year after adjuvant chemotherapy in patients with HER2-positive early breast cancer: A 4-year follow-up of a randomised controlled trial. *The Lancet Oncology*. 2011;**12**(3):236-244
- [11] Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. *The New England Journal of Medicine*. 2005;**353**(16):1659-1672
- [12] Seidman AD, Fornier MN, Esteva FJ, Tan L, Kaptain S, Bach A, et al. Weekly trastuzumab and paclitaxel therapy for metastatic breast cancer with analysis of efficacy by HER2 immunophenotype and gene amplification. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology*. 2001;**19**(10):2587-2595
- [13] Vogel CL, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, et al. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology*. 2002;**20**(3):719-726
- [14] Xia W, Mullin RJ, Keith BR, Liu LH, Ma H, Rusnak DW, et al. Anti-tumor activity of GW572016: A dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. *Oncogene*. 2002;**21**(41):6255-6263
- [15] Ryan Q, Ibrahim A, Cohen MH, Johnson J, Ko CW, Sridhara R, et al. FDA drug approval summary: Lapatinib in combination with capecitabine for previously treated metastatic breast cancer that overexpresses HER-2. *The Oncologist*. 2008;**13**(10):1114-1119
- [16] Verma S, Ewer MS. Is cardiotoxicity being adequately assessed in current trials of cytotoxic and targeted agents in breast cancer? *Annals of Oncology: Official Journal of the European Society for Medical Oncology*. 2011;**22**(5):1011-1018
- [17] Press MF, Cordon-Cardo C, Slamon DJ. Expression of the HER-2/neu proto-oncogene in normal human adult and fetal tissues. *Oncogene*. 1990;**5**(7):953-962
- [18] Ross JS, Fletcher JA, Bloom KJ, Linette GP, Stec J, Clark E, et al. HER-2/neu testing in breast cancer. *American Journal of Clinical Pathology*. 2003;**120**(Suppl):S53-S71
- [19] Kallioniemi OP, Kallioniemi A, Kurisu W, Thor A, Chen LC, Smith HS, et al. ERBB2 amplification in breast cancer analyzed by fluorescence in situ hybridization. *Proceedings of the National Academy of Sciences of the United States of America*. 1992;**89**(12):5321-5325
- [20] Iqbal N, Iqbal N. Human Epidermal Growth Factor Receptor 2 (HER2) in cancers: Overexpression and therapeutic implications. *Molecular Biology International*. 2014; **852748**:2014
- [21] Hubbard SR, Till JH. Protein tyrosine kinase structure and function. *Annual Review of Biochemistry*. 2000;**69**:373-398

- [22] Carpenter G. Receptors for epidermal growth factor and other polypeptide mitogens. *Annual Review of Biochemistry*. 1987;**56**:881-914
- [23] Roskoski R Jr. ErbB/HER protein-tyrosine kinases: Structures and small molecule inhibitors. *Pharmacological Research*. 2014;**87**:42-59
- [24] Sierke SL, Cheng K, Kim HH, Koland JG. Biochemical characterization of the protein tyrosine kinase homology domain of the ErbB3 (HER3) receptor protein. *The Biochemical Journal*. 1997;**322**(Pt 3):757-763
- [25] Kim HH, Vijapurkar U, Hellyer NJ, Bravo D, Koland JG. Signal transduction by epidermal growth factor and heregulin via the kinase-deficient ErbB3 protein. *The Biochemical Journal*. 1998;**334**(Pt 1):189-195
- [26] Carrera AC, Alexandrov K, Roberts TM. The conserved lysine of the catalytic domain of protein kinases is actively involved in the phosphotransfer reaction and not required for anchoring ATP. *Proceedings of the National Academy of Sciences of the United States of America*. 1993;**90**(2):442-446
- [27] Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell*. 2010;**141**(7):1117-1134
- [28] Zaczek A, Brandt B, Bielawski KP. The diverse signaling network of EGFR, HER2, HER3 and HER4 tyrosine kinase receptors and the consequences for therapeutic approaches. *Histology and Histopathology*. 2005;**20**(3):1005-1015
- [29] Wilson KJ, Gilmore JL, Foley J, Lemmon MA, Riese DJ 2nd. Functional selectivity of EGF family peptide growth factors: Implications for cancer. *Pharmacology & Therapeutics*. 2009;**122**(1):1-8
- [30] Rubin I, Yarden Y. The basic biology of HER2. *Annals of Oncology: Official Journal of the European Society for Medical Oncology*. 2001;**12**(Suppl 1):S3-S8
- [31] Burgess AW, Cho HS, Eigenbrot C, Ferguson KM, Garrett TP, Leahy DJ, et al. An open-and-shut case? Recent insights into the activation of EGF/ErbB receptors. *Molecular Cell*. 2003;**12**(3):541-552
- [32] Sliwkowski MX. Ready to partner. *Nature Structural Biology*. 2003;**10**(3):158-159
- [33] Chen H, Xu CF, Ma J, Eliseenkova AV, Li W, Pollock PM, et al. A crystallographic snapshot of tyrosine trans-phosphorylation in action. *Proceedings of the National Academy of Sciences of the United States of America*. 2008;**105**(50):19660-19665
- [34] Paul MK, Mukhopadhyay AK. Tyrosine kinase—Role and significance in Cancer. *International Journal of Medical Sciences*. 2004;**1**(2):101-115
- [35] Moasser MM. The oncogene HER2: Its signaling and transforming functions and its role in human cancer pathogenesis. *Oncogene*. 2007;**26**(45):6469-6487
- [36] Nguyen B, Keane MM, Johnston PG. The biology of growth regulation in normal and malignant breast epithelium: From bench to clinic. *Critical Reviews in Oncology/Hematology*. 1995;**20**(3):223-236

- [37] Hemmings BA, Restuccia DF. PI3K-PKB/Akt pathway. *Cold Spring Harbor Perspectives in Biology*. 2012;**4**(9):a011189
- [38] Santarpia L, Lippman SM, El-Naggar AK. Targeting the MAPK-RAS-RAF signaling pathway in cancer therapy. *Expert Opinion on Therapeutic Targets*. 2012;**16**(1):103-119
- [39] Baulida J, Kraus MH, Alimandi M, Di Fiore PP, Carpenter G. All ErbB receptors other than the epidermal growth factor receptor are endocytosis impaired. *The Journal of Biological Chemistry*. 1996;**271**(9):5251-5257
- [40] Bertelsen V, Stang E. The mysterious ways of ErbB2/HER2 trafficking. *Membranes*. 2014;**4**(3):424-446
- [41] Tzahar E, Waterman H, Chen X, Levkowitz G, Karunakaran D, Lavi S, et al. A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. *Molecular and Cellular Biology*. 1996;**16**(10):5276-5287
- [42] Atalay G, Cardoso F, Awada A, Piccart MJ. Novel therapeutic strategies targeting the epidermal growth factor receptor (EGFR) family and its downstream effectors in breast cancer. *Annals of Oncology: Official Journal of the European Society for Medical Oncology*. 2003;**14**(9):1346-1363
- [43] Graus-Porta D, Beerli RR, Daly JM, Hynes NE. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. *The EMBO Journal*. 1997;**16**(7):1647-1655
- [44] Gutierrez C, Schiff R. HER2: Biology, detection, and clinical implications. *Archives of Pathology & Laboratory Medicine*. 2011;**135**(1):55-62
- [45] Way TD, Lin JK. Role of HER2/HER3 co-receptor in breast carcinogenesis. *Future Oncology*. 2005;**1**(6):841-849
- [46] Nahta R, Yuan LX, Zhang B, Kobayashi R, Esteva FJ. Insulin-like growth factor-I receptor/human epidermal growth factor receptor 2 heterodimerization contributes to trastuzumab resistance of breast cancer cells. *Cancer Research*. 2005;**65**(23):11118-11128
- [47] Bononi A, Agnoletto C, De Marchi E, Marchi S, Patergnani S, Bonora M, et al. Protein kinases and phosphatases in the control of cell fate. *Enzyme Research*. 2011;**2011**:329098
- [48] Arteaga CL. Epidermal growth factor receptor dependence in human tumors: More than just expression? *The Oncologist*. 2002;**7**(Suppl 4):31-39
- [49] Brooks AN, Kilgour E, Smith PD. Molecular pathways: Fibroblast growth factor signaling: A new therapeutic opportunity in cancer. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research*. 2012;**18**(7):1855-1862
- [50] Nishikawa R, Ji XD, Harmon RC, Lazar CS, Gill GN, Caveness WK, et al. A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity. *Proceedings of the National Academy of Sciences of the United States of America*. 1994;**91**(16):7727-7731

- [51] Ocana A, Vera-Badillo F, Seruga B, Templeton A, Pandiella A, Amir E. HER3 overexpression and survival in solid tumors: A meta-analysis. *Journal of the National Cancer Institute*. 2013;**105**(4):266-273
- [52] Roskoski R Jr. Vascular endothelial growth factor (VEGF) signaling in tumor progression. *Critical Reviews in Oncology/Hematology*. 2007;**62**(3):179-213
- [53] Stern DF. Tyrosine kinase signalling in breast cancer: ErbB family receptor tyrosine kinases. *Breast Cancer Research: BCR*. 2000;**2**(3):176-183
- [54] Hendriks BS, Opresko LK, Wiley HS, Lauffenburger D. Quantitative analysis of HER2-mediated effects on HER2 and epidermal growth factor receptor endocytosis: Distribution of homo- and heterodimers depends on relative HER2 levels. *The Journal of Biological Chemistry*. 2003;**278**(26):23343-23351
- [55] Huang G, Chantry A, Epstein RJ. Overexpression of ErbB2 impairs ligand-dependent downregulation of epidermal growth factor receptors via a post-transcriptional mechanism. *Journal of Cellular Biochemistry*. 1999;**74**(1):23-30
- [56] Wang Z, Zhang L, Yeung TK, Chen X. Endocytosis deficiency of epidermal growth factor (EGF) receptor-ErbB2 heterodimers in response to EGF stimulation. *Molecular Biology of the Cell*. 1999;**10**(5):1621-1636
- [57] Lenferink AE, Pinkas-Kramarski R, van de Poll ML, van Vugt MJ, Klapper LN, Tzahar E, et al. Differential endocytic routing of homo- and hetero-dimeric ErbB tyrosine kinases confers signaling superiority to receptor heterodimers. *The EMBO Journal*. 1998;**17**(12):3385-3397
- [58] Waterman H, Yarden Y. Molecular mechanisms underlying endocytosis and sorting of ErbB receptor tyrosine kinases. *FEBS Letters*. 2001;**490**(3):142-152
- [59] Hendriks BS, Wiley HS, Lauffenburger D. HER2-mediated effects on EGFR endosomal sorting: Analysis of biophysical mechanisms. *Biophysical Journal*. 2003;**85**(4):2732-2745
- [60] Timms JF, White SL, O'Hare MJ, Waterfield MD. Effects of ErbB-2 overexpression on mitogenic signalling and cell cycle progression in human breast luminal epithelial cells. *Oncogene*. 2002;**21**(43):6573-6586
- [61] Wolff AC, Hammond ME, Hicks DG, Dowsett M, McShane LM, Allison KH, et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology*. 2013;**31**(31):3997-4013
- [62] Varshney D, Zhou YY, Geller SA, Alsabeh R. Determination of HER-2 status and chromosome 17 polysomy in breast carcinomas comparing HercepTest and PathVysion FISH assay. *American Journal of Clinical Pathology*. 2004;**121**(1):70-77
- [63] Hanna W, Kahn HJ, Trudeau M. Evaluation of HER-2/neu (erbB-2) status in breast cancer: From bench to bedside. *Modern Pathology: An Official Journal of the United States and Canadian Academy of Pathology, Inc*. 1999;**12**(8):827-834

- [64] Yildiz-Aktas IZ, Dabbs DJ, Bhargava R. The effect of cold ischemic time on the immunohistochemical evaluation of estrogen receptor, progesterone receptor, and HER2 expression in invasive breast carcinoma. *Modern Pathology: An Official Journal of the United States and Canadian Academy of Pathology, Inc.* 2012;**25**(8):1098-1105
- [65] Middleton LP, Price KM, Puig P, Heydon LJ, Tarco E, Sneige N, et al. Implementation of American Society of Clinical Oncology/College of American Pathologists HER2 Guideline Recommendations in a tertiary care facility increases HER2 immunohistochemistry and fluorescence in situ hybridization concordance and decreases the number of inconclusive cases. *Archives of Pathology & Laboratory Medicine.* 2009;**133**(5):775-780
- [66] Tsuda H, Sasano H, Akiyama F, Kurosumi M, Hasegawa T, Osamura RY, et al. Evaluation of interobserver agreement in scoring immunohistochemical results of HER-2/neu (c-erbB-2) expression detected by HercepTest, Nichirei polyclonal antibody, CB11 and TAB250 in breast carcinoma. *Pathology International.* 2002;**52**(2):126-134
- [67] Zhao J, Wu R, Au A, Marquez A, Yu Y, Shi Z. Determination of HER2 gene amplification by chromogenic in situ hybridization (CISH) in archival breast carcinoma. *Modern Pathology: An Official Journal of the United States and Canadian Academy of Pathology, Inc.* 2002;**15**(6):657-665
- [68] Hoang MP, Sahin AA, Ordonez NG, Sneige N. HER-2/neu gene amplification compared with HER-2/neu protein overexpression and interobserver reproducibility in invasive breast carcinoma. *American Journal of Clinical Pathology.* 2000;**113**(6):852-859
- [69] Sanderson MP, Dempsey PJ, Dunbar AJ. Control of ErbB signaling through metalloprotease mediated ectodomain shedding of EGF-like factors. *Growth Factors.* 2006;**24**(2):121-136
- [70] Carney WP, Leitzel K, Ali S, Neumann R, Lipton A. HER-2/neu diagnostics in breast cancer. *Breast Cancer Research: BCR.* 2007;**9**(3):207
- [71] Lam L, McAndrew N, Yee M, Fu T, Tchou JC, Zhang H. Challenges in the clinical utility of the serum test for HER2 ECD. *Biochimica et Biophysica Acta.* 2012;**1826**(1):199-208
- [72] Moelans CB, de Weger RA, Van der Wall E, van Diest PJ. Current technologies for HER2 testing in breast cancer. *Critical Reviews in Oncology/Hematology.* 2011;**80**(3):380-392
- [73] Hicks DG, Tubbs RR. Assessment of the HER2 status in breast cancer by fluorescence in situ hybridization: A technical review with interpretive guidelines. *Human Pathology.* 2005;**36**(3):250-261
- [74] Ross JS, Slodkowska EA, Symmans WF, Pusztai L, Ravdin PM, Hortobagyi GN. The HER-2 receptor and breast cancer: Ten years of targeted anti-HER-2 therapy and personalized medicine. *The Oncologist.* 2009;**14**(4):320-368
- [75] Tse CH, Hwang HC, Goldstein LC, Kandalaft PL, Wiley JC, Kussick SJ, et al. Determining true HER2 gene status in breast cancers with polysomy by using alternative chromosome 17 reference genes: Implications for anti-HER2 targeted therapy. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology.* 2011;**29**(31):4168-4174

- [76] Varga Z, Noske A, Ramach C, Padberg B, Moch H. Assessment of HER2 status in breast cancer: Overall positivity rate and accuracy by fluorescence in situ hybridization and immunohistochemistry in a single institution over 12 years: A quality control study. *BMC Cancer*. 2013;**13**:615
- [77] Bartlett JM, Going JJ, Mallon EA, Watters AD, Reeves JR, Stanton P, et al. Evaluating HER2 amplification and overexpression in breast cancer. *The Journal of Pathology*. 2001;**195**(4):422-428
- [78] Paik S, Bryant J, Tan-Chiu E, Romond E, Hiller W, Park K, et al. Real-world performance of HER2 testing--National Surgical Adjuvant Breast and bowel project experience. *Journal of the National Cancer Institute*. 2002;**94**(11):852-854
- [79] Press MF, Hung G, Godolphin W, Slamon DJ. Sensitivity of HER-2/neu antibodies in archival tissue samples: Potential source of error in immunohistochemical studies of oncogene expression. *Cancer Research*. 1994;**54**(10):2771-2777
- [80] Tubbs RR, Pettay JD, Roche PC, Stoler MH, Jenkins RB, Grogan TM. Discrepancies in clinical laboratory testing of eligibility for trastuzumab therapy: Apparent immunohistochemical false-positives do not get the message. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology*. 2001;**19**(10):2714-2721
- [81] Yeh IT. Measuring HER-2 in breast cancer. Immunohistochemistry, FISH, or ELISA? *American Journal of Clinical Pathology*. 2002;**117**(Suppl):S26-S35
- [82] Bhargava R, Lal P, Chen B. Chromogenic in situ hybridization for the detection of HER-2/neu gene amplification in breast cancer with an emphasis on tumors with borderline and low-level amplification: Does it measure up to fluorescence in situ hybridization? *American Journal of Clinical Pathology*. 2005;**123**(2):237-243
- [83] Yaziji H, Goldstein LC, Barry TS, Werling R, Hwang H, Ellis GK, et al. HER-2 testing in breast cancer using parallel tissue-based methods. *Journal of the American Medical Association*. 2004;**291**(16):1972-1977
- [84] Ridolfi RL, Jamehdor MR, Arber JM. HER-2/neu testing in breast carcinoma: A combined immunohistochemical and fluorescence in situ hybridization approach. *Modern Pathology: An Official Journal of the United States and Canadian Academy of Pathology, Inc*. 2000;**13**(8):866-873
- [85] Dowsett M, Hanna WM, Kockx M, Penault-Llorca F, Ruschoff J, Gutjahr T, et al. Standardization of HER2 testing: Results of an international proficiency-testing ring study. *Modern Pathology: An Official Journal of the United States and Canadian Academy of Pathology, Inc*. 2007;**20**(5):584-591
- [86] Furrer D, Jacob S, Caron C, Sanschagrin F, Provencher L, Diorio C. Validation of a new classifier for the automated analysis of the human epidermal growth factor receptor 2 (HER2) gene amplification in breast cancer specimens. *Diagnostic Pathology*. 2013;**8**:17
- [87] Stevens R, Almanaseer I, Gonzalez M, Caglar D, Knudson RA, Ketterling RP, et al. Analysis of HER2 gene amplification using an automated fluorescence in situ hybridization signal enumeration system. *The Journal of Molecular Diagnostics: JMD*. 2007;**9**(2):144-150

- [88] Tubbs RR, Pettay JD, Swain E, Roche PC, Powell W, Hicks DG, et al. Automation of manual components and image quantification of direct dual label fluorescence in situ hybridization (FISH) for HER2 gene amplification: A feasibility study. *Applied Immunohistochemistry & Molecular Morphology: AIMM*. 2006;**14**(4):436-440
- [89] Furrer D, Sanschagrin F, Jacob S, Diorio C. Advantages and disadvantages of technologies for HER2 testing in breast cancer specimens. *American Journal of Clinical Pathology*. 2015;**144**(5):686-703
- [90] Penault-Llorca F, Bilous M, Dowsett M, Hanna W, Osamura RY, Ruschoff J, et al. Emerging technologies for assessing HER2 amplification. *American Journal of Clinical Pathology*. 2009;**132**(4):539-548
- [91] Gong Y, Gilcrease M, Sneige N. Reliability of chromogenic in situ hybridization for detecting HER-2 gene status in breast cancer: Comparison with fluorescence in situ hybridization and assessment of interobserver reproducibility. *Modern Pathology: An Official Journal of the United States and Canadian Academy of Pathology, Inc*. 2005;**18**(8):1015-1021
- [92] Isola J, Tanner M, Forsyth A, Cooke TG, Watters AD, Bartlett JM. Interlaboratory comparison of HER-2 oncogene amplification as detected by chromogenic and fluorescence in situ hybridization. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research*. 2004;**10**(14):4793-4798
- [93] Gupta D, Middleton LP, Whitaker MJ, Abrams J. Comparison of fluorescence and chromogenic in situ hybridization for detection of HER-2/neu oncogene in breast cancer. *American Journal of Clinical Pathology*. 2003;**119**(3):381-387
- [94] Garcia-Caballero T, Grabau D, Green AR, Gregory J, Schad A, Kohlwes E, et al. Determination of HER2 amplification in primary breast cancer using dual-colour chromogenic in situ hybridization is comparable to fluorescence in situ hybridization: A European multicentre study involving 168 specimens. *Histopathology*. 2010;**56**(4):472-480
- [95] Lambros MB, Natrajan R, Reis-Filho JS. Chromogenic and fluorescent in situ hybridization in breast cancer. *Human Pathology*. 2007;**38**(8):1105-1122
- [96] Hwang CC, Pintye M, Chang LC, Chen HY, Yeh KY, Chein HP, et al. Dual-colour chromogenic in-situ hybridization is a potential alternative to fluorescence in-situ hybridization in HER2 testing. *Histopathology*. 2011;**59**(5):984-992
- [97] Powell RD, Pettay JD, Powell WC, Roche PC, Grogan TM, Hainfeld JF, et al. Metallographic in situ hybridization. *Human Pathology*. 2007;**38**(8):1145-1159
- [98] Bartlett JM, Campbell FM, Ibrahim M, Wencyk P, Ellis I, Kay E, et al. Chromogenic in situ hybridization: A multicenter study comparing silver in situ hybridization with FISH. *American Journal of Clinical Pathology*. 2009;**132**(4):514-520
- [99] Francis GD, Jones MA, Beadle GF, Stein SR. Bright-field in situ hybridization for HER2 gene amplification in breast cancer using tissue microarrays: Correlation between chromogenic (CISH) and automated silver-enhanced (SISH) methods with patient outcome. *Diagnostic Molecular Pathology: The American Journal of Surgical Pathology, Part B*. 2009;**18**(2):88-95

- [100] Nitta H, Hauss-Wegrzyniak B, Lehrkamp M, Murillo AE, Gaire F, Farrell M, et al. Development of automated brightfield double in situ hybridization (BDISH) application for HER2 gene and chromosome 17 centromere (CEN 17) for breast carcinomas and an assay performance comparison to manual dual color HER2 fluorescence in situ hybridization (FISH). *Diagnostic Pathology*. 2008;**3**:41
- [101] Schiavon BN, Jasani B, de Brot L, Vassallo J, Damascena A, Cirullo-Neto J, et al. Evaluation of reliability of FISH versus brightfield dual-probe in situ hybridization (BDISH) for frontline assessment of HER2 status in breast cancer samples in a community setting: Influence of poor tissue preservation. *The American Journal of Surgical Pathology*. 2012;**36**(10):1489-1496
- [102] Bartlett JM, Campbell FM, Ibrahim M, O'Grady A, Kay E, Faulkes C, et al. A UK NEQAS ISH multicenter ring study using the Ventana HER2 dual-color ISH assay. *American Journal of Clinical Pathology*. 2011;**135**(1):157-162
- [103] Franchet C, Filleron T, Cayre A, Mounie E, Penault-Llorca F, Jacquemier J, et al. Instant-quality fluorescence in-situ hybridization as a new tool for HER2 testing in breast cancer: A comparative study. *Histopathology*. 2014;**64**(2):274-283
- [104] Matthiesen SH, Hansen CM. Fast and non-toxic in situ hybridization without blocking of repetitive sequences. *PLoS One*. 2012;**7**(7):e40675
- [105] Ohlschlegel C, Kradolfer D, Hell M, Jochum W. Comparison of automated and manual FISH for evaluation of HER2 gene status on breast carcinoma core biopsies. *BMC Clinical Pathology*. 2013;**13**:13
- [106] Cronin M, Pho M, Dutta D, Stephans JC, Shak S, Kiefer MC, et al. Measurement of gene expression in archival paraffin-embedded tissues: Development and performance of a 92-gene reverse transcriptase-polymerase chain reaction assay. *The American Journal of Pathology*. 2004;**164**(1):35-42
- [107] Noske A, Loibl S, Darb-Esfahani S, Roller M, Kronenwett R, Muller BM, et al. Comparison of different approaches for assessment of HER2 expression on protein and mRNA level: Prediction of chemotherapy response in the neoadjuvant GeparTrio trial (NCT00544765). *Breast Cancer Research and Treatment*. 2011;**126**(1):109-117
- [108] Jacquemier J, Spyrtos F, Esterni B, Mozziconacci MJ, Antoine M, Arnould L, et al. SISH/CISH or qPCR as alternative techniques to FISH for determination of HER2 amplification status on breast tumors core needle biopsies: A multicenter experience based on 840 cases. *BMC Cancer*. 2013;**13**:351
- [109] Merkelbach-Bruse S, Wardelmann E, Behrens P, Losen I, Buettner R, Friedrichs N. Current diagnostic methods of HER-2/neu detection in breast cancer with special regard to real-time PCR. *The American Journal of Surgical Pathology*. 2003;**27**(12):1565-1570
- [110] Nistor A, Watson PH, Pettigrew N, Tabiti K, Dawson A, Myal Y. Real-time PCR complements immunohistochemistry in the determination of HER-2/neu status in breast cancer. *BMC Clinical Pathology*. 2006;**6**:2
- [111] Esteva FJ, Yu D, Hung MC, Hortobagyi GN. Molecular predictors of response to trastuzumab and lapatinib in breast cancer. *Nature Reviews Clinical Oncology*. 2010;**7**(2):98-107