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Chapter 2

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

Daniela Furrer, Claudie Paquet, Simon Jacob and Caroline Diorio

Additional information is available at the end of the chapter

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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 \( \text{HER2} \) 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 \( \text{HER2} \) 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 \( \text{HER2} \) (also known as \( \text{erb-b2 receptor tyrosine kinase 2 (ERBB2)} \) 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 \( \text{HER2} \) gene and up to 20,000 HER2 receptors) [18], whereas in HER2-positive breast cancer cells, there is an increase in the number of \( \text{HER2} \) 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 (TGFα), 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 autocrine 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 11–25% of tumor cells), 3+ (strong, complete membrane staining in >25% of tumor cells).
staining in ≤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 is 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 centromere 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 have been approved by the FDA for the automated determination of HER2 gene amplification: the Metafer (MetaSystems, Altussheim, 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]. Hereafter, 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 aneusomy, 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 monocolor 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 aneusomy 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**

The authors have no other declarations.

**Acronyms and abbreviations**

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<th>Acronym</th>
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<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>GRB7</td>
<td>Growth factor receptor bound protein 7</td>
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<tr>
<td>ESR1</td>
<td>Estrogen Receptor 1</td>
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<td>EGFR</td>
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IHC  Immunohistochemistry
FISH  Fluorescence *in situ* 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
TGFα  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  *in situ* hybridization
CISH  Chromogenic *in situ* hybridization
SISH  Silver-enhanced *in situ* 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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