Soluble isoforms of EGFR family in breast cancer: Advances and clinical relevance (Review)

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Abstract. Breast cancer (BrCa) is the leading cause of cancer death among women worldwide. The epidermal growth factor receptor (EGFR) family, consisting of EGFR/erythroblastic leukemia viral (v-erbB) oncogene homolog (ErbB)1, human epidermal growth factor receptor (HER)2/ErbB2, HER3/ErbB3 and HER4/ErbB4, serves a pivotal role in BrCa pathogenesis. In addition to the membrane-bound forms, soluble isoforms of these receptors have emerged as biologically and clinically significant players in BrCa. These soluble variants are produced either through alternative splicing or proteolytic cleavage of the extracellular domain. Functionally, soluble EGFR family members can act as decoy receptors or interfere with receptor homo- or heterodimerization, ultimately disrupting downstream signaling cascades and contributing to the dysregulated growth and survival of BrCa cells. Notably, soluble EGFR and HER2 are detectable in the serum of patients with BrCa, and their serum levels fluctuate during disease progression and treatment. These dynamic serum fluctuations possess strong prognostic and predictive potential. This review summarized the current understanding of soluble EGFR family members in BrCa, including their mechanisms of generation and biological functions. The clinical relevance of soluble EGFR and the shed extracellular domain of HER2 in BrCa is addressed, with an emphasis on their emerging roles as diagnostic biomarkers and promising therapeutic targets.

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Abbreviations: aa, amino acid; ADAM, a disintegrin and metalloproteinase; ADC, antibody-drug conjugate; BrCa, breast cancer; CA15-3: cancer antigen 15-3; CM, conditioned media; CNR, copy number ratio; CTC, circulating tumor cell; ctDNA, circulating tumor DNA; Cyt, cytosolic C-terminus; DFS, disease-free survival; ECD, extracellular domain; EGFR, epidermal growth factor receptor; ER, estrogen receptor; ErbB, erythroblastic leukemia viral (v-erbB) oncogene homolog; F1,6BP: fructose 1,6 bisphosphate; ^{18}F-FDG, fluorine-18 fluorodeoxyglucose; 4-CTF, HER4 C-terminal fragment; HAIA, human anti-animal immunoglobulin antibody; HAMA, human anti-mouse antibody; HER, human epidermal growth factor receptor; HRG, heregulin; ICD, intracellular domain; I8/9/12, intron 8/9/12/15; JM, juxtamembrane; LBD, ligand-binding domain; mAb, monoclonal antibody; mBrCa, metastatic BrCa; MMP, matrix metalloproteinase; MW, molecular weight; NRG, neuregulin; ORR, objective response rate; OS, overall survival; PFS, progression-free survival; polyA, polyadenylation; PET/CT, positron emission tomography computed tomography; sEGFR, soluble EGFR; sHER2, soluble HER2; sHER3, soluble HER3; RTK, receptor tyrosine kinase; TACE, necrosis factor-α converting enzyme; T-DM1, trastuzumab-emtansine; TGF-α, transforming growth factor-α; TKI, tyrosine kinase inhibitor; TNBC, triple-negative BrCa; TTP, time to progression

Key words: BrCa, soluble EGFR, soluble HER2, herstatin, soluble HER3, soluble HER4, extracellular domain

Contents

1. Introduction

Breast cancer (BrCa) is the most commonly diagnosed cancer among women worldwide, with ~2.3 million new cases in 2022. Although improvements have been made in the survival rates, BrCa remains the leading cause of cancer death in women, with an estimated 666,000 deaths worldwide in 2022 (1). It is therefore critical to identify novel potential biomarkers and therapeutic targets for BrCa.

The epidermal growth factor receptor (EGFR) family serves a crucial role in the onset and progression of BrCa (2). This family is also referred to as human epidermal growth factor receptor (HER) or erythroblastic leukemia viral (v-erbB) oncogene homolog (ErbB) family (3). The EGFR family comprises four structurally related members, including EGFR/HER1/ErbB1, HER2/ErbB2/Neu, HER3/ErbB3 and HER4/ErbB4. Each member consists of a signal peptide, an extracellular domain (ECD), a single transmembrane domain, a juxtamembrane segment, an intracellular tyrosine kinase domain and a C-terminal tail. The ECDs, comprised of two ligand-binding subdomains (I and III) and two structural cysteine-rich subdomains (II and IV) (Fig. 1) (4), undergo extensive N-glycosylation, which modulates receptor structure, function and therapeutic response (5).

Thus far, 11 peptide growth factors have been identified as EGFR family ligands, namely: Amphiregulin, betacellulin, EGF, epigen, epiregulin, heparin-binding EGF-like growth factor, neuregulin (NRG) 1-4 and transforming growth factor-α (TGF-α). EGFR, HER3 and HER4 have different ligand specificities, but HER2 has no known ligand due to its defective ligand-binding domain (LBD). HER3 has defective kinase activity (Fig. 1) (6). Ligand binding induces homo- or heterodimerization, autophosphorylation and receptor activation, which in turn activate multiple signaling pathways, including PI3K/Akt, Ras/Raf/MAPK, JAK/STAT and PLCγ1/PKC. These signaling pathways regulate cell proliferation, apoptosis, migration and differentiation. Aberrant activation of the EGFR family is associated with aggressive tumor behavior and poor prognosis (2).

Each EGFR family member possesses truncated soluble isoforms, which retain the extracellular LBD but lack the transmembrane segment and intracellular domain (ICD). These soluble isoforms are generated through either alternative mRNA splicing or proteolytic cleavage of their transmembrane counterparts (7,8). Once being released into the extracellular space, soluble receptors can interact with ligands or membrane-bound receptors, thereby modulating signaling pathways initiated by full-length receptors (8). Soluble isoforms of the EGFR family have been detected in BrCa cells and tissues, and their clinical potential has attracted significant research interest in recent years (7,9). This review summarized the generation and biological functions of soluble EGFR family members in BrCa, highlighting the clinical relevance of soluble EGFR and shed HER2 ECD in BrCa, and discussed their potential as biomarkers and therapeutic targets.

2. Soluble EGFR isoforms

EGFR, the prototype of the EGFR family, was first identified in the late 1970s and early 1980s (10). The human *EGFR* gene, located on chromosome 7p11.2 and spanning ~200 kb, contains 28 exons that encode a 170 kDa protein (11). EGFR upregulation is documented in up to 60% of patients with BrCa (12,13) and is associated with poor clinical outcomes (14). Alternative mRNA splicing and proteolytic cleavage produce multiple soluble EGFR (sEGFR) isoforms, which have been observed in BrCa cells and tissues (7).

Generation of sEGFR by alternative mRNA splicing. Alternative splicing and utilization of alternative polyadenylation (polyA) signals give rise to multiple EGFR transcripts across various species, including humans (11). Among these, the 10-10.6, 5.6-6.3 and 2.8-3.2 kb transcripts are the most predominant (15-18). The two longer transcripts, generated through alternative splicing, encode the full-length EGFR and are detected in human adult and fetal tissues, as well as in a wide range of cancer cell lines (11,15-18). By contrast, the 2.8-3.2 kb transcript results from a translocation between the 5' region of the EGFR gene and an unidentified region of genomic DNA (19), and is only found in human placenta and carcinoma cell lines with *EGFR* gene amplification, such as epidermoid A431carcinoma and breast MDA-MB-468 cancer cells (11,15-18,20). This transcript encodes a truncated 110 kDa sEGFR termed p110 sEGFR or EGFR isoform D (21), which was first observed in A431 cells over four decades ago (15,18,22). The p110 sEGFR encompasses extracellular subdomains I-III and subdomain IV up to amino acid (aa) residue 603 of full-length EGFR plus 78 unique C-terminal residues (Fig. 2A). The p110 sEGFR is a plasma membrane-associated protein and can be secreted. The transcription of p110 sEGFR in human BrCa cells is upregulated by EGFR ligands (EGF and NRG) and phorbol myristate acetate through the Ras/Raf/MAPK signaling pathway and PKC, respectively. However, it remains to be investigated whether EGFR ligands and phorbol myristate acetate trigger the secretion of p110 sEGFR (21).

Generation of the EGFR ECD by proteolytic cleavage. EGFR is not a classical metalloprotease substrate; therefore, the ectodomain shedding of full-length EGFR by proteolytic cleavage is rare. However, a 110 kDa shed EGFR (p110 EGFR ECD) is detected in the conditioned media (CM) of MDA-MB-468 cells. The p110 EGFR ECD has the same EGFR sequence up to aa 625 and shares the same sequence with p110 sEGFR (681 aa residues) up to aa 603 (Fig. 2A). The production of the p110 EGFR ECD is stimulated by EGF, TGF-a and PKC. Treatment of MDA-MB-468 cells with various matrix metalloprotease inhibitors revealed that EGFR shedding is mediated by a secreted matrix metalloproteinase (MMP) family members rather than a disintegrin and metalloproteinase (ADAM) 17/tumor necrosis factor-α converting enzyme (TACE) (23).

A 90 kDa (p90) sEGFR, part of the alternatively spliced p110 sEGFR, is ectopically expressed in CHO cells and found to be associated with the plasma membrane through hydrophobic interactions. Proteolytic cleavage of this p90 sEGFR by metalloprotease TACE liberates an 80 kDa (p80) sEGFR, and this process is inhibited by fibronectin and stimulated by an anti-α5/β1 integrin antibody (24). It was demonstrated that EFGR shedding is a regulated process. In human HaCaT keratinocyte cells, metalloprotease and calcium flux activate the ectodomain shedding of cellular and exosomal EGFR, producing a 150 kDa (p150) sEGFR and a less common 100 kDa (p100) sEGFR. Further studies indicate that ADAM17 does not stimulate shedding of p150 sEGFR (25). The shed sEGFR isoforms are associated with exosomes, probably via dimerization with full-length EGFR on the exosome surface (25). Whether BrCa cells produce p80, p100 and p150 shed sEGFR isoforms remains to be investigated.

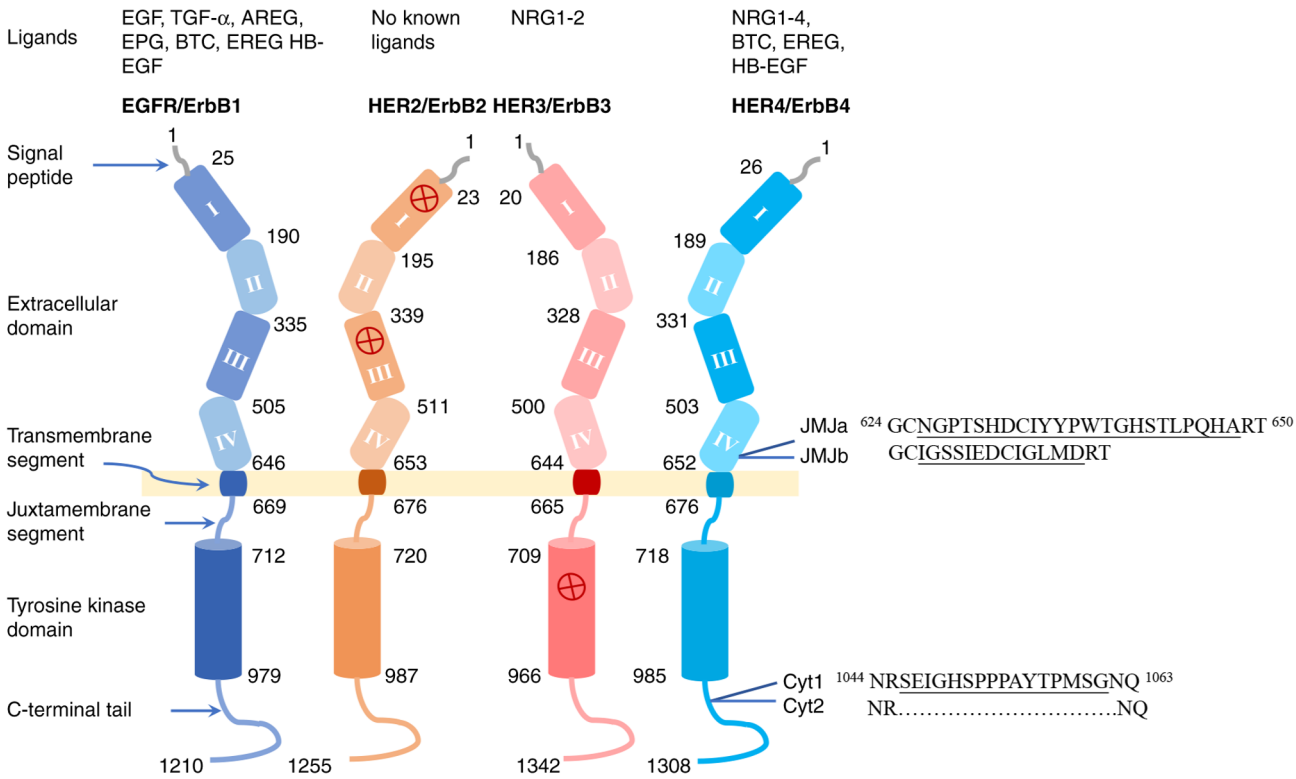

Figure 1. The structure and ligands of the EGFR family. The extracellular domains consist of subdomains I, II, III and IV. The ligand-binding domain of HER2 and the tyrosine kinase domain of HER3 are defective. HER4 has two alternative splicing sites, the JM splicing site results in isoforms JMa and JMb, and the Cyt splicing site results in isoforms Cyt1 and Cyt2. The amino acid sequence differences among HER4 isoforms are indicated on the right. The numbers indicate the initial amino acid residue of each (sub)domain except for the last residues of the receptors (3,6). The red cross symbols indicate functional deficient domains. AREG, amphiregulin; BTC, betacellulin; EPG, epigen; EREG, epiregulin; HB-EGF, heparin-binding EGF-like growth factor; NRG, neuregulin; JM, juxtamembrane; Cyt, C-terminal region; EGFR, epidermal growth factor receptor;

Two EGFR-targeted therapeutic antibodies, cetuximab and panitumumab, inhibit sEGFR shedding (24), indicating that EGFR shedding may result in malignancy, and blocking this process may represent a potential therapeutic strategy for cancer.

Biological functions of sEGFR isoforms. The shed sEGFR isoforms can function as decoy receptors by binding and sequestering ligands, such as EGF (24) and betacellulin (25) or form heterodimers with full-length EGFR at the cell surface or on exosome surface (25,26), thereby reducing ligand availability and inhibiting EGFR kinase activity. sEGFR isoforms inhibit the proliferation and migration of cancer cells, potentially by blocking the internalization of full-length EGFR (7). Nevertheless, the effects of sEGFR isoforms on canonical EGFR signaling pathways, including PI3K/AKT, Ras/Raf/MAPK and JAK/STAT, remain to be investigated.

Cell-surface EGFR has been implicated in aerobic glycolysis and immune escape in triple-negative BrCa (TNBC) cells. In EGF-treated TNBC cells, EGFR phosphorylates and inactivates pyruvate kinase, which catalyzes the last glycolytic step, leading to the accumulation of glycolytic intermediates. Accumulated fructose 1,6 bisphosphate (F1,6BP) directly binds to EGFR and enhances its activity, resulting in increased extracellular level of lactate, which in turn inhibits local cytotoxic T-cell activity. It was suggested that the EGF/EGFR/F1,6BP signaling axis promotes TNBC cells to evade immunosurveillance (27). Further research to determine whether sEGFR modulates the immune response of TNBC cells via the EGF/EGFR/F1,6BP signaling axis is warranted. Information on tumor glycolytic activity can be obtained using fluorine-18 fluorodeoxyglucose (^{18}F-FDG) positron emission tomography computed tomography (PET/CT) (28). Analyzing the relationship between ^{18}F-FDG PET/CT metabolic parameters, tumor-infiltrating lymphocytes in BrCa specimens and serum sEGFR levels in patients with BrCa may offer insights into the influence of sEGFR on glycolysis and immune responses in BrCa.

Diagnostic and prognostic potential of sEGFR. sEGFR is detected in the serum of patients with BrCa (29,30). The concentration of serum sEGFR is in the range of ng/ml, some of which may be lower than the detection limit. Recently, Zhang *et al* (29) developed a nanoproteomics approach that integrates aptamer-modified metal-organic frameworks with LC-MS/MS for the enrichment and quantitative analysis of sEGFR family proteins in serum samples of patients with various malignant tumors, including breast, lung, gastric and colorectal cancers. This method demonstrates high sensitivity and specificity, with a detection limit as low as 1.00 nM for these proteins. Wignarajah *et al* (30) developed an electrochemical immunosensor to measure serum sEGFR concentration in patients with BrCa, achieving a quantification limit of 98 pg/ml. Therefore, the development of novel detection technologies may improve the detection limit of serum sEGFRs.

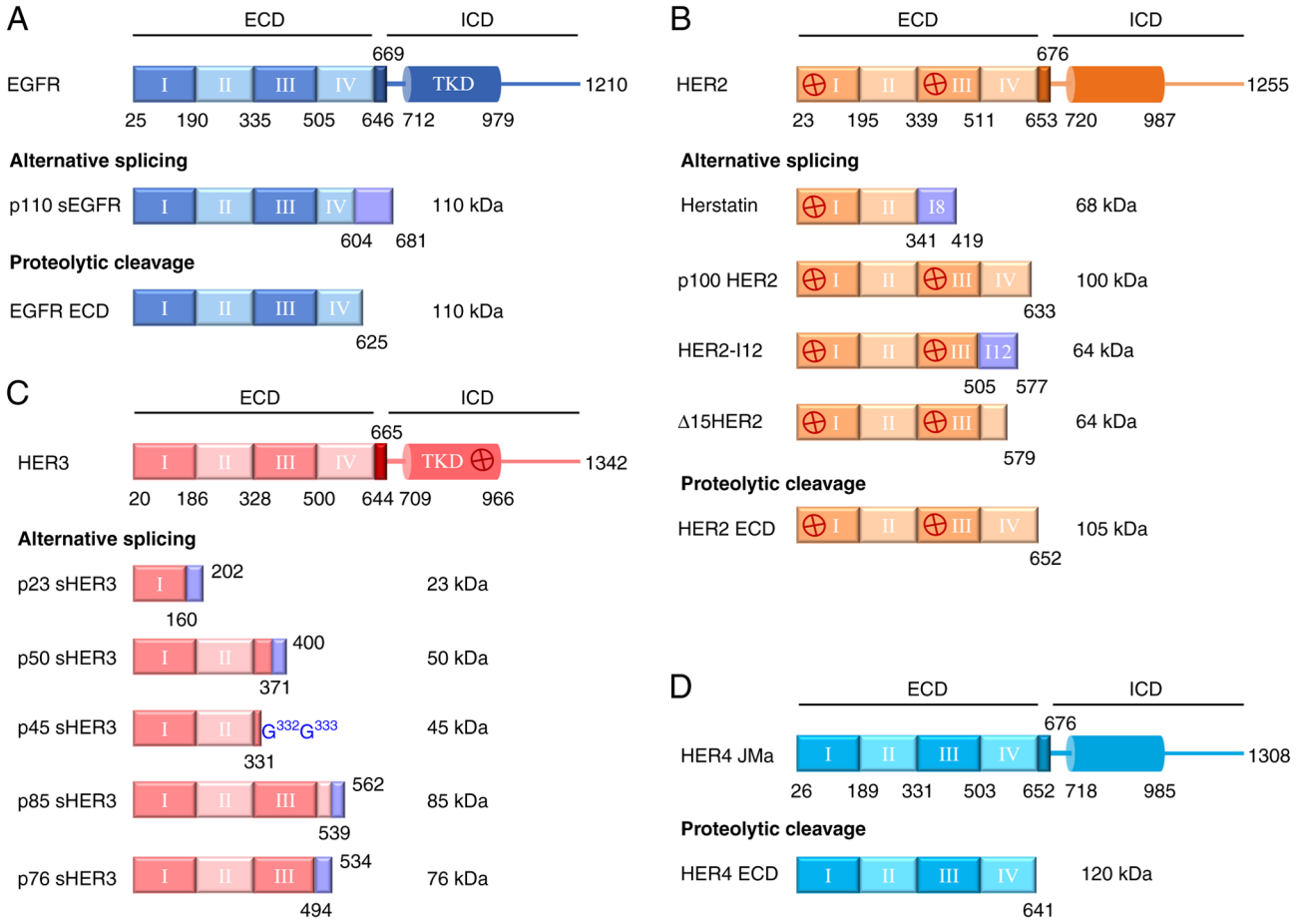

Figure 2. Soluble isoforms of EGFR family. (A) sEGFR isoforms. Alternative splicing produces p110 sEGFR with a novel 78 C-terminal amino acid residues. (B) Soluble HER2 isoforms. Retention of I8 during alternative splicing results in herstatin, which contains a unique 79 C-terminal residues encoded by I8. Retention of I12 results in HER2-I12 with a unique 73 C-terminal residues. (C) sHER3 isoforms produced via alternative splicing. p23 sHER3 contains a unique 43 C-terminal residues. Retention of I9 results in p50 sHER3 with a novel C-terminal region (30 residues), retention of I8 and I9 results in p45 sHER3 with two unique C-terminal glycine residues, retention of I13 results in p85 sHER3 with a unique 23 C-terminal residues and retention of I12 and I13 results in p76 sHER3 with a unique 41 C-terminal residues. (D) Soluble HER4 isoform produced by proteolytic cleavage. The numbers indicate the initial amino acid residue of each (sub)domain except for the last residues of the proteins (23,51,54,55,57,67,95,96,108). The red cross symbols indicate functional deficient domains. I, intron; EGFR, epidermal growth factor receptor; JM, juxtamembrane; sEGFR, soluble EGFR; ECD, extracellular domain; ICD, intracellular domain; HER, human epidermal growth factor receptor; sHER3, soluble HER3.

Although sEGFR is generated from full-length EGFR, serum sEGFR levels are not concordant with EGFR expression in primary BrCa (31,32). Patients may have serum sEGFR levels comparable to those of healthy controls (12,33,34) or significantly lower (14,29,35). Furthermore, serum sEGFR levels vary greatly across different patient subgroups. The estrogen receptor (ER)-positive subgroup of early-stage patients with BrCa has markedly elevated sEGFR levels compared with the ER-negative subgroup and healthy controls (36). Notably, EGFR-positive patients may have significantly lower sEGFR levels compared with EGFR-negative patients (29). Furthermore, clinical treatment such as chemotherapy and endocrine therapy may decrease (33,37) or not significantly change (34) serum sEGFR levels in patients with metastatic BrCa (mBrCa). Proteomic analysis of serum proteins and breast tissues from patients with BrCa and healthy individuals may help identify the sources of serum sEGFR and understand the mechanisms regulating its release into the circulation.

EGFR upregulation has been considered a poor prognostic factor in BrCa for decades (14); however, the serum sEGFR levels in patients with BrCa do not correlate with clinical outcomes. In patients with early BrCa, a significant decrease of serum sEGFR level from pre- to post-surgery is significantly associated with a worse prognosis (38), and a lower preoperative serum sEGFR level (<60.3 ng/ml) is correlated with a shorter overall survival (OS) and invasive disease-free survival (DFS) (39). In patients with locally advanced or mBrCa, a higher serum sEGFR level is associated with longer OS (12). By contrast, a low baseline serum sEGFR level is associated with shorter survival or reduced treatment response to, particularly in patients with ER-positive tumors (40). These data suggest a positive correlation between serum sEGFR and survival. By contrast, de Araujo *et al* (14) reported that patients with TNBC with lower serum sEGFR level have a markedly higher median OS compared with those with higher sEGFR level. Other studies showed that pretreatment serum sEGFR level in patients with early BrCa is not correlated with DFS or histopathological response (38,41), and baseline serum sEGFR level in patients with locally advanced or mBrCa is not associated with progression-free survival (PFS), OS, response rate or clinical benefit rate (40). These observations imply that serum sEGFR levels may correlate positively, negatively or not correlate with clinical outcomes in patients with BrCa.

Altogether, serum sEGFR levels are not associated with EGFR expression in BrCa and may change dynamically during treatment. Furthermore, serum sEGFR levels do not correlate with the clinical outcomes. Therefore, serum sEGFR levels do not consistently demonstrate diagnostic or prognostic value in BrCa.

3. Soluble HER2 isoforms

The human *HER2* gene was identified in the 1980s by three independent groups and named as *neu* (42), *HER2* (43) or *c-erbB-2* (44). The *HER2* gene is located on chromosome 17q21 (43,45,46) and encodes a 185 kDa protein (43). Although HER2 has a defective LBD, it can form homo- or heterodimers with other EGFR family members in a ligand-independent manner. HER2 homodimers are generally less potent compared with heterodimers. In BrCa, the most common heterodimer is HER2/HER3, which has strong potency in activating downstream signaling pathways (47).

The *HER2* gene is amplified and upregulated in 20% of primary BrCa (8). HER2 status, (positive, equivocal or negative) is determined based on assessment of gene amplification (via *in situ* hybridization) and/or protein upregulation (by immunohistochemistry), as per the American Society of Clinical Oncology and College of American Pathologists guidelines. Patients with HER2-positive BrCa meet eligibility criteria for HER2-targeted therapies (48). Approximately 17.4% of patients with BrCa are classified as HER2-positive (49), which is generally associated with a more aggressive tumor and worse prognosis (50). Soluble HER2 (sHER2) isoforms, produced via either alternative mRNA splicing or proteolytic cleavage, have been detected in human BrCa cells and tissues (8).

Generation of sHER2 by alternative mRNA splicing

Herstatin. Herstatin (dimercept), a 68 kDa secreted protein, is translated from an alternatively spliced 2.6 kb HER2 transcript that retains intron 8 (I8). Herstatin consists of the extracellular subdomains I and II (340 aa residues) of full-length HER2, followed by a unique 79 C-terminal residues encoded by I8 (Fig. 2B) (51). Overexpression of splicing factor hnRNP A1 in HER2-positive human breast SKBR3 cancer cells promotes retention of I8. It increases herstatin protein levels, whereas overexpression of the splicing factor SRSF1 promotes the splicing of I8 and increases endogenous HER2. This study implied that hnRNP A1 and SRSF1 are essential regulators of I8 splicing (52).

p100 HER2. A 2.3 kb truncated HER2 transcript identified in HER2-positive human breast BT474 and SKBR3 cells is found to be generated by reading through a splice donor site located at the 5'end of I15. The 5' 2.1 kb segment of the 2.3 kb transcript is identical to the 5'end of the full-length 4.6 kb HER2 transcript, and the remaining segment contains the retained 5'splice donor site of I5, the in-frame stop codon and the polyA signal. The 2.3 kb transcript encodes a 100 kDa truncated protein that consists of almost the entire ECD of full-length HER2 (633 aa residues) except for the C-terminal 20 residues (Fig. 2B) (53,54). Splicing factor SRSF3 suppresses the inclusion of I15 and decreases the level of p100 HER2 (55).

HER2-I12. The HER2-I12 transcript arises from the retention of I12 (361 bp) during alternative splicing of the HER2 pre-mRNA. It encodes a truncated 64 kDa protein due to the introduction of a premature stop codon 212 nucleotides downstream of the start of I12. HER2-I12 protein comprises extracellular subdomains I-III (504 aa residues) of full-length HER2, followed by a unique 73 C-terminal residues (Fig. 2B) (56).

Δ15HER2. Wan *et al* (57) reported that a splice-switching oligonucleotide induces the skipping of exon 15 in SKBR3 cells, generating a HER2 mRNA lacking exon 15 (Δ15HER2). Exon skipping generates a stop codon in exon 16, leading to the synthesis of a truncated soluble protein that retains 579 out of 652 aa residues of the HER2 ECD sequence (Fig. 2B). Further investigation is warranted to determine whether the Δ15HER2 transcript is naturally produced in BrCa cells and tissues.

Generation of HER2 ECD by proteolytic cleavage. Proteolytic cleavage of HER2 results in the release of a 105 kDa protein and the production of a membrane-anchored 95 kDa protein. The 105 kDa protein (652 aa) comprises the ECD of full-length HER2 (HER2 ECD) (Fig. 2B), while the 95 kDa protein consists of the transmembrane segment and the ICD of full-length HER2 (HER2 ICD). HER2 ECD is detected in the CM of human BrCa cell lines with high HER2 expression (58,59) as well as in the serum of patients with BrCa (60). Shedding of HER2 ECD is regulated by the metalloprotease ADAM 10. Both ADAM10 siRNA and a specific ADAM10 inhibitor of reduce HER2 ECD shedding of in SKBR3 and BT474 cells (58,61). Furthermore, high levels of ADAM10 expression is observed in 80% of patients with BrCa with high levels of HER2 ECD, whereas negative or weak expression of ADAM10 is observed in patients with BrCa with no serum HER2 ECD (61). This indicated that ADAM10 is involved in HER2 ECD shedding in BrCa. Matriptase, a membrane-associated serine protease, has been reported to cleave the ECD of phosphorylated HER2 at Arg558 and Arg599, and such cleavage is inhibited by hepatocyte growth factor activator inhibitor 1 in MDA-MB-231 cells (62). It is unclear whether matriptase cleaves HER2 *in vivo* and releases the ECD fragment into the circulation.

In addition to ADAM10, HER2-targeting reagents also regulate HER2 ECD levels in cancer cells. Lapatinib, a dual tyrosine kinase inhibitor (TKI) of EGFR and HER2, induces the accumulation of inactive HER2 at the cell surface of SKBR3 cells and markedly increases the shedding of HER2 ECD. Gefitinib, an EGFR TKI, moderately upregulates HER2 ECD release (63). Trastuzumab (Herceptin), a recombinant humanized HER2 monoclonal antibody (mAb), binds to the extracellular subdomain IV of HER2, which also contains the proteolytic cleavage site (64). The binding of trastuzumab blocks protease access to HER2, leading to a significant down-regulation of HER2 ECD level (65). These studies suggest that HER2 ECD levels may change dynamically during treatment, but the underlying mechanism is not well understood.

Biological functions of sHER2 isoforms

Herstatin. Herstatin binds to the extracellular subdomain II (cysteine-rich) of HER2 by its C-terminal region encoded by I8 (66). Herstatin-HER2 interaction retains HER2 in the ER and decreases cell-surface HER2 level (67). Herstatin also binds to other receptor tyrosine kinases (RTKs), including

EGFR and IGF-1R. The binding affinity of herstatin for HER2 and EGFR is comparable, ~10-fold that of IGF-1R (68). Strikingly, I8 encoded peptide alone also binds multiple RTKs, including EGFR, HER2, HER4 and IGF-1R, with 3-4-fold lower affinity for HER2 and EGFR compared with herstatin (51,68,69). The mutation R371I in I8 eliminates its receptor-binding capacity (68). Structural analysis reveals that isolated I8 is intrinsically disordered, and $I8^{R371I}$ is prone to aggregation, leading to loss of receptor-binding activity (70).

Herstatin functions as an autoinhibitor of HER2. Mechanistically, herstatin interferes with the homo- and heterodimerization of HER2 (51,71), inhibits HER2 tyrosine phosphorylation, prevents transactivation of HER3 by HER2 (51,71,72) and downregulates protein levels of HER3 and HER4 (72). Furthermore, herstatin blocks the activation of EGFR, Akt and Erk1/2 induced by EGFR ligands, including EGF, heregulin (HRG), and TGF-α (51,71-73), thereby inhibiting proliferation and colony formation of HER2-overexpressing cells (51,67,71,73). Herstatin restores tamoxifen sensitivity in HER2-overexpressing BrCa cells (72). Notably, the I8 peptide also inhibits the viability of HER2-positive BrCa cells in a dose-dependent manner, and cells with higher HER2 expression are more sensitive to I8 (69). These results indicate that both herstatin and I8 are tumor suppressive, probably by disrupting HER2-containing dimers.

The expression of herstatin is low in HER2-overexpressing SKBR3 and BT474 cells, whereas high levels of herstatin and low levels of full-length HER2 are detected in nontumorigenic cells (51). Furthermore, herstatin is present in mast cells and epithelial cells in noncancerous breast tissues, but is absent in ~75% of BrCa tissues (74). These observations further demonstrate that herstatin is tumor suppressive.

Given that herstatin blocks HER2 dimerization (51,71), clinical treatments aimed at disrupting HER2 dimer may be expected to be efficient for patients with herstatin-negative BrCa but inefficient for herstatin-positive tumors (74).

p100 HER2. p100 HER2 can be secreted or distributed within the perinuclear cytoplasm. Upregulation of p100 HER2 in HER2-overexpressing BrCa cells results in resistance to the growth inhibitory effects of anti-HER2 mAbs. The underlying mechanism is not well understood; intracellular p100 HER2 may block antibody-mediated inactivation of HER2 signaling cascades (54). Overexpression of p100 HER2 in BrCa cells with low levels of HER2 inhibits cell proliferation and HRG-induced colony formation, whereas downregulation of endogenous p100 HER2 in HER2-overexpressing cells significantly enhances EGF-induced colony formation. Mechanistically, p100 HER2 prevents tyrosine phosphorylation of HER2 and HER4, and blocks activation of Erk1/2 (53). This study implicates that p100 HER2 inhibits growth factor-induced proliferation of BrCa cells.

Gastric tumors at higher stages tend to have lower expression of p100 HER2 (53); however, the mRNA levels of p100 HER2 in BrCa samples correlate with the development of lymph node and bone marrow metastasis (75). These conflicting data indicate that the role of p100 HER2 in human cancer may depend on tumor type or other confounding variables.

HER2-I12. HER2-I12 mRNA is detected in both HER2-positive and HER2-negative human BrCa cell lines, but the expression level is not concordant with HER2 levels. In addition, HER2-I12 mRNA is detected in normal breast and BrCa tissues, with the highest expression in HER2-positive BrCa followed by HER2-negative BrCa and normal tissues (56). Endogenous HER2-I12 protein expression has not been reported yet. In MCF7 cells, heterologous HER2-I12 proteins are detected in the membrane fraction, implying that HER2-I12 may bind to the plasma membrane or dimerize with other EGFR family members. Similar to full-length HER2, HER2-I12 activates both Ras/Raf/MAPK and PI3K/Akt signaling pathways in MCF7 cells, and promotes cell proliferation, migration and invasion (56). Hence, HER2-I12 may have pro-oncogenic effects, in contrast to tumor-suppressive activity of herstatin.

Δ15HER2. Δ15HER2 can interact with membrane-bound HER2 and HER3. Addition of exogenous Δ15HER2 proteins to human BrCa cells downregulates HER2 and HER3, decreases phosphorylation of HER2, HER3 and Akt, and specifically kills HER2-overexpressing BrCa cells (57). Δ15HER2 thus has tumor-suppressive activity similar to that of herstatin.

HER2 ECD. HER2 ECD cDNA-transfected human ovarian SKOV3 carcinoma cells with HER2 overexpression release high levels of HER2 ECD into the media, while EGFR and HER2 are expressed at equal levels in transfected and control cells. The released HER2 ECD significantly inhibits HER2-driven cell proliferation both *in vitro* and *in vivo*. HER2 ECD forms heterodimers with EGFR, HER2 and HER3, thereby preventing phosphorylation of EGFR, HER2, Akt and ERK1/2 in both transfected cells and tumor xenografts (76). Lapatinib markedly promotes basal shedding of HER2 ECD to inhibit HER2-driven BrCa cell growth (63). Trastuzumab inhibits phosphorylation of HER3 and Akt in HER2-overexpressing BrCa cells (64) and suppresses HER2-driven tumor growth, while HER2 ECD enhances the inhibitory effects of trastuzumab on BrCa cells and tumor xenografts (64,76). Notably, lapatinib-resistant BrCa cells exhibit significantly reduced levels of HER2 ECD relative to their lapatinib-sensitive parental counterparts. By contrast, trastuzumab-resistant BrCa cells display nearly undetectable levels of HER2 ECD compared with trastuzumab-sensitive parental cells. Lapatinib and trastuzumab synergistically inhibit BrCa cell growth, whereas lapatinib treatment significantly enhances HER2 ECD shedding, which sensitizes trastuzumab-resistant cells to lapatinib-induced growth inhibition (63). Mechanistically, HER2 ECD may facilitate the accumulation of trastuzumab on HER2-positive tumor cells, thereby inhibiting tumor growth (76). These findings suggest a feedback mechanism in which HER2-targeted therapies modulate HER2 ECD shedding, and HER2 ECD levels are negatively correlated with therapeutic resistance. Further studies are needed to confirm whether HER2 ECD can serve as a potential predictor of response or resistance to the HER2-targeted drugs such as trastuzumab and lapatinib.

It has been reported that HER2-positive BrCa cells can attract immune cells to the tumor microenvironment (TME) by secreting chemokines and cytokines. Tumor-infiltrating immune cells, such as T, B and natural killer cells, interact with HER2-positive BrCa cells and suppress tumor growth and progression. However, HER2-positive BrCa cells also express immune checkpoint molecules (such as programmed death-ligand 1) and attract immunosuppressive cells such

as regulatory T cells, myeloid-derived suppressor cells and tumor-associated macrophages, thereby contributing to immune evasion in HER2-positive BrCa (47). The role of sHER2 isoforms in modulating immune cells in the TME remains unknown.

Altogether, sHER2 isoforms dimerize with EGFR (herstatin and HER2 ECD), HER2 (herstatin, Δ15HER2 and HER2 ECD) and HER3 (Δ15HER2 and HER2 ECD), which in turn prevents tyrosine phosphorylation of EGFR (HER2 ECD), HER2 (herstatin, p100 HER2, Δ15HER2 and HER2 ECD), HER3 (herstatin, Δ15HER2) and HER4 (p100 HER2), leading to inhibition of EGFR downstream signaling pathways PI3K/Akt (herstatin, Δ15HER2 and HER2 ECD) and Ras/Raf/MAPK (herstatin, p100 HER2 and HER2 ECD). By contrast, HER2-I12 activates both PI3K/Akt and Ras/Raf/MAPK signaling pathways *in vitro*, but the underlying mechanism is not well understood. Herstatin, p100 HER2, Δ15HER2 and HER2 ECD are therefore tumor suppressive, whereas HER2-I12 may be pro-oncogenic. Future studies may investigate the influence of sHER2 isoforms on immune cells in the TME.

Diagnostic and prognostic potential of HER2 ECD. HER2 ECD is released into circulation and can be measured in the serum or plasma of patients with cancer. Enzyme-linked immunosorbent assay is an attractive approach for detecting serum HER2 ECD, with a sensitivity of 0.5 ng/ml (77). In the past decade, nanomaterials-based immunosensors (antibody/affibody-based) and aptasensors (aptamer-based) have been developed for rapid, sensitive and cost-effective detection of serum HER2 ECD (78), with detection limits ranging from 10 to 80 pg/ml (30,79). Raman spectroscopy is a powerful analytical technique for quantifying biomolecules in serum samples. Ma *et al* (80) developed a method to detect serum surface-enhanced Raman scattering (SERS) signals by optimizing a composite silver nanoparticles PSi Bragg reflector SERS substrate, achieving an accuracy of 95%. Serum Raman spectroscopy combined with deep learning algorithms can reach an accuracy rate of 90% (81).

Patients with BrCa have significantly higher serum HER2 ECD levels compared with healthy controls (29,82), and HER2-positive vs. HER2-negative patients have markedly higher HER2 ECD levels (29,61). Furthermore, a high level of HER2 ECD in patients with advanced BrCa is significantly correlated with visceral and liver metastasis (83), as well as the number of metastatic sites (84). Notably, HER2 ECD levels are correlated with HER2 expression in BrCa (29,61,79,83,85-87). Serum HER2 ECD, therefore, might be a surrogate marker for HER2 to identify patients with BrCa eligible to anti-HER2 treatment.

An increase in serum HER2 ECD level is observed in patients with higher clinical stages (82), metastasis and recurrence (29,88). According to the US Food and Drug Administration, the cut-off value for an elevated HER2 ECD level is ≤15 ng/ml (48). Elevated HER2 ECD levels have been documented to correlate with aggressive clinicopathologic features; 30.6% of patients with BrCa, 5% of patients with benign breast tumors and 4% of healthy controls exhibit elevated HER2 ECD levels (61), 20-87% of patients with mBrCa vs. 4-18.5% of primary patients with BrCa display elevated serum HER2 ECD (50,60,89), 82% of patients at stage III vs. 30% of patients at stages I-II have elevated HER2 ECD, and 43.2% of patients with HER2-positive mBrCa vs. 16.1% of patients with HER2-positive primary BrCa have elevated HER2 ECD (29). Nevertheless, 36.9% of patients with HER2-positive BrCa have HER2 ECD levels <15 ng/ml, and the concordance between elevated HER2 ECD and HER2-overexpression is 63.1% (61). Therefore, elevated HER2 ECD may not be a reliable diagnostic marker for early-stage BrCa and HER2 status. Instead, it is more closely associated with advanced tumor stage and metastasis. Consequently, early screening for elevated HER2 ECD may enable more sensitive detection of mBrCa.

As aforementioned, the *HER2* gene is amplified in 20% of primary BrCa cases (8). Qui *et al* (84) measured the HER2 copy number ratios (HER2-CNRs) in cell-free DNA purified from peripheral blood by using droplet digital PCR. They found that plasma HER2-CNRs positively correlate with serum HER2 ECD levels in HER2-positive patients with BrCa (88), but a slight decrease in plasma HER2-CNR and an marked decrease in serum HER2-ECD level are detected in patients with HER2-positive BrCa treated with neoadjuvant chemotherapy (84). Notably, a discrepancy is observed between HER2-CNR and HER2 ECD level in HER2-positive patients with advanced BrCa treated with the antibody-drug conjugate (ADC) trastuzumab-emtansine (T-DM1). HER2-CNR is invariably reduced under T-DM1 pressure, whereas HER2 ECD is elevated in 81% of patients. *In vitro* experiments reveal that HER2 ECD is preferentially released by dying BrCa cells treated with T-DM1. Furthermore, HER2 ECD elevation is associated with longer PFS than HER2 ECD reduction. Patients with fast disease progression undergo a concerted reduction in HER2-CNR and HER2 ECD (90). This study suggests that assessing both HER2-CNR and HER2 ECD during treatment may help tailor anti-HER2 therapies, particularly ADCs active on HER2-CNR-low and HER2 ECD-low BrCa.

The prognostic significance of altered HER2 ECD levels in BrCa has been extensively studied. Primary patients with BrCa with elevated HER2 ECD levels tend to have shorter PFS (61), worse distant-metastasis-free survival, poor BrCa-specific survival (91), lower survival rate (82) and higher recurrence incidence (92) compared with those with low HER2 ECD levels. In patients with advanced BrCa, high baseline HER2 ECD levels are associated with shorter time to progression (TTP) but higher objective response rate (ORR) (83). In patients with invasive BrCa with HER2 overexpression, elevated HER2 ECD levels are significantly associated with worse OS; furthermore, non-metastatic and metastatic subgroups with elevated HER2 ECD levels at diagnosis have significantly worse DFS and shorter PFS, respectively, compared with those with low HER2 ECD levels (93). Zhang *et al* (94) systematically reviewed 23 studies (8,231 patients) that investigated the prognostic value of HER2 ECD in patients with BrCa, and found that an elevated baseline serum HER2 ECD level is significantly correlated with worse OS, DFS, PFS and TTP. Compared with baseline HER2 ECD levels, significant decreases (>20%) have been observed in patients during treatment (29,83,84,89,95-97). The decreased HER2 ECD is strongly associated with prolonged TTP, higher ORR (83) and complete response (97). Patients with mBrCa with HER2 ECD levels ≥28.3 ng/ml compared with those

with levels <28.3 ng/ml have significantly worse DFS, whereas those with a >20% decrease of HER2 ECD have longer DFS after receiving chemotherapy (29). These studies indicate that elevated HER2 ECD is associated with poor prognosis in patients with BrCa, whereas a significant decrease in HER2 ECD level during treatment indicates a favorable treatment response and improved prognosis.

Nevertheless, conflicting results have been reported. An elevated HER2 ECD level in patients with HER2-positive mBrCa is significantly associated with a higher response rate before initiation of trastuzumab treatment, but PFS and OS are not linked to elevated HER2 ECD (96).

To evaluate the effects of elevated HER2 ECD on the efficacy of different treatment modalities, Wu *et al* (50) conducted a systematic review and meta-analysis of 40 studies (l12,229 patients) conducted between 2001 and 2021. The results showed that elevated serum HER2 ECD levels are significantly associated with worse PFS, DFS and OS in patients treated with chemotherapy or trastuzumab. Patients receiving endocrine therapy with elevated sHER2 ECD have markedly shorter PFS and OS compared with those with low sHER2 ECD, and those receiving adjuvant therapy with elevated sHER2 ECD vs. those with low sHER2 ECD exhibit notably reduced DFS and OS. However, serum HER2 ECD levels are not correlated with PFS and DFS in patients treated with TKIs such as lapatinib (50). The meta-analysis suggested that elevated HER2 ECD is an unfavorable prognostic factor in BrCa and may guide selection of appropriate anti-HER2 therapy for HER2-positive BrCa.

Altogether, HER2 ECD may serve as a surrogate marker for HER2, as HER2 ECD levels correlate with HER2 overexpression in BrCa. Elevated HER2 ECD levels correlate with tumor aggressiveness and poor prognosis in patients with BrCa, whereas a significant decrease in HER2 ECD levels during treatment indicates better prognosis. Therefore, assessment of HER2 ECD levels at diagnosis and during treatment may help to select appropriate anti-HER2 therapy for HER2-positive BrCa.

Despite being proposed as a promising biomarker for detecting recurrence and monitoring disease status in HER2-positive BrCa, the use of serum HER2 ECD as a stand-alone test for diagnosis or major treatment decisions remains challenging. First, there is no universal cut-off to distinguish 'normal' from 'elevated' HER2 ECD levels. Although most assays adopt the FDA-recommended threshold of 15 ng/ml (83), differences among assay platforms preclude generalizing a single cut-off across all methods. Additionally, baseline HER2 ECD levels may vary by ethnicity; for example, Asian patients tend to exhibit significantly higher levels compared with other ethnicities (65). Second, serum factors such as human anti-animal immunoglobulin antibodies (HAIAs) or more commonly human anti-mouse antibodies (HAMAs) can interfere with immunological assays. In ELISA-based tests, HAIA/HAMA may bind to capture antibodies and sterically block signal antibody binding, producing false-negative results. They can also crosslink capture and signal antibodies, leading to false-positive readings (98). Third, only ~30% of patients with BrCa exhibit elevated HER2 ECD levels (61), limiting its utility as a broad diagnostic tool. Fourth, moderately elevated HER2 EDC levels (up to 50 ng/ml) have been reported in several non-cancerous conditions, including liver disease, pre-eclampsia and chronic heart failure. Notably, 40-60% of patients with non-cancerous hepatic diseases exhibit increased HER2 ECD (65). While optimizing threshold values could improve test specificity and sensitivity, and removing immunoglobulins or using blocking reagents may reduce HAIA/HAMA interference, the fundamental constraints, such as the detection of elevated HER2 ECD in ~30% of patients with BrCa and in up to 60% of non-cancerous liver diseases, continue to limit the clinical application of serum HER2 ECD testing for patients with BrCa.

Since the clinical utility of HER2 ECD in patients with BrCa remains controversial, the combination of HER2 ECD with other tumor markers has been investigated for prognosis and therapeutic response monitoring. In patients with primary untreated BrCa, the subgroup with high serum levels of both HER2 ECD and cancer antigen 15-3 (CA15-3) has a worse DFS compared with the other subgroups (HER2 ECD^{high} + $CA15\text{-}3^{normal}$, HER2 ECD^{normal} + $CA15\text{-}3^{high}$, HER2 ECD^{normal} + $CA15\text{-}3^{normal}$) (99). In patients with mBrCa, the average decrease of serum HER2 ECD, CA15-3 and carcinoembryonic antigen with a threshold of >10% appears to be the best parameter to distinguish patients with progressive disease from other patients (89), whereas high serum levels of both HER2 ECD and MMP-9 discriminate patients with brain metastasis (100). The prognostic values of these HER2 ECD-based multi-biomarker panels need to be further evaluated in large-scale studies.

Liquid biopsy is a minimally invasive approach that analyzes circulating tumor components such as circulating tumor cells (CTCs), circulating tumor DNA (ctDNA) and exosomes from body fluids. The presence of ≥5 CTCs in 7.5 ml blood is a poor prognostic marker in mBrCa. Patients (n=253) with ≥5 CTCs are more likely to have elevated HER2 ECD, which is associated with worse survival. However, HER2 ECD status does not provide additional prognostic information when CTC status is available (101). Another study (n=68) demonstrates that high serum HER2 ECD levels are significantly associated with the absolute number of CTCs and HER2-positive CTCs (102). ctDNA is released into the bloodstream by dying tumor cells and carries key genetic information that reflects the genomic alterations of the tumor. In a systematic review and meta-analysis encompassing 4,264 patients with mBrCa, specific genomic alterations in ctDNA (*TP53* and *ESR1*) are significantly associated with worse survival outcomes (103). Exosomal proteins reflect the physiological state of the originating cell and serve an essential role in intercellular communication in cancer. Exosomal fibronectin, developmental endothelial locus-1 and survivin-2B have been proposed as potential biomarkers for early detection of BrCa (104). Future studies integrating HER2 ECD levels and other liquid biopsy data may uncover novel prognostic and therapeutic avenues for BrCa.

4. Soluble HER3 isoforms

The human *HER3* gene was first identified in 1989. It is located on chromosome 12q13 and is expressed as a 6.2 kb transcript that encodes a 180 kDa protein. HER3 is expressed in a variety of normal epithelial tissues and in certain human

BrCa cell lines (105); 30% of primary BrCa and 60% of brain metastases express HER3. In comparison, phosphorylated HER3 is detected in 37% of primary BrCa and up to 85% of brain metastases (106), indicating a role of HER3 in the progression of BrCa. HER3 is upregulated in various types of cancer, including BrCa, but HER3 upregulation alone is not sufficient to cause carcinogenesis. HER3 has defective kinase activity, so HER3 homodimers are not functional, but HER3 heterodimers, especially HER2/HER3 heterodimers, serve crucial roles in the onset and progression of BrCa (107). Soluble HER3 (sHER3) isoforms generated by alternative mRNA splicing have been identified in BrCa cells.

Generation of sHER3 by alternative mRNA splicing. A 1.4 kb alternate HER3 transcript was initially identified in gastric cancer cell lines and mucosa (108). A further study showed that this transcript is expressed in human breast, ovarian and gastric carcinoma cell lines, as well as in normal placental tissues (109). The 1.4 kb transcript encodes a 23 kDa predicted protein of 183 aa residues; the first 140 aa residues are identical to the extracellular subdomain I of HER3 with a unique 43 carboxy-terminal residues (Fig. 2C). However, this protein is only detected in cell lysate but not in the CM when ectopically expressed in fibroblasts (108,109).

Of note, 5 years after the identification of the 1.4 kb HER3 transcript, four more alternate HER3 transcripts (1.6, 1.7, 2.1 and 2.3 kb) were discovered in human ovarian, breast and gastric carcinoma cell lines. These transcripts are produced by readthrough of an intron and use of an alternative polyA signal within this intron. The 1.6 kb transcript retains I9, which contains an alternative polyA signal, and encodes a 42 kDa predicted protein. The first 351 aa residues of this protein are identical to the extracellular subdomains I, II, and a portion of subdomain III of HER3 followed by 30 unique C-terminal residues (Fig. 2C). The 1.7 kb transcript retains I8 (introducing an in-frame stop codon) and I9, it encodes a 34 kDa predicted protein (312 aa residues) comprised of the extracellular subdomains I, II, and a portion of subdomain III of HER3 followed by two unique glycine residues (Fig. 2C). The 2.1 kb transcript retains I13 (containing an alternative polyA signal) and encodes a 60 kDa predicted protein, which consists of the extracellular subdomains I, II, III, and a portion of subdomain IV of HER3 (519 aa residues) followed by 24 unique C-terminal residues (Fig. 2C). The 2.3 kb transcript retains I12 (introducing an in-frame stop codon) and I13 and predicts a 57 kDa protein. The first 474 aa residues of this protein are identical to the extracellular subdomains I, II and III of HER3, followed by 41 unique C-terminal residues (Fig. 2C). Transfection of cDNAs corresponding to these four transcripts into fibroblasts results in the expression of truncated proteins with the apparent MWs of 50, 45, 85 and 76 kDa. The first protein is only detected in cell lysates, while the latter three are detected in both cell lysates and CM. N-glycosylation may cause the differences between the apparent and calculated MWs (109). In primary cultures of ovarian carcinoma samples, a 90 kDa protein in the CM is detected by an antibody directed against the LBD of HER3. This protein may correspond to one of the three secreted HER3 proteins in fibroblasts, and the differences in size may result from a higher level of N-glycosylation. However, this protein may also arise from a proteolytic cleavage of HER3. The identity of this 90 kDa protein and the functions of the truncated HER3 proteins remain to be investigated (109).

To our knowledge, the shedding of HER3 ECD has not yet been reported. Matriptase, hepsin and prostasin, the membrane-associated serine proteases, have been reported to cleave the ECD of HER3 in HEK293 cells (62). It is unknown whether these proteases cleave HER3 in BrCa cells and tissues.

Biological functions of sHER3 isoforms. The 45 kDa (p45) and 85 kDa (p85) sHER3 proteins, the products of the 1.7 and 2.1 transcripts, respectively, inhibit HER3 phosphorylation stimulated by HRGα and HRGβ, but p85 sHER3 is more effective than p45 sHER3. p85 sHER3 binds to HRG and competitively inhibits high-affinity binding of HRG to HER2/HER3 heterodimers on BrCa cell surface (110). However, another study showed that p85 sHER3 associates with cell-surface HER2 and HER3 in BrCa cells (111). p85 sHER3 blocks HRG-induced phosphorylation of EGFR, HER2, HER3 and HER4 in BrCa cells (110,111), interferes the formation of HER3-containing heterodimers especially HER2/HER3 (111), prevents the association of HER3 with the regulatory subunit p85 of PI3K (110) and suppresses the activation of ERK1/2 and Akt, leading to inhibition of HRG stimulated proliferation of BrCa cells (110,111). These results suggest that p85 sHER3 negatively regulates HRG-stimulated signal transduction in human malignancies, but the underlying mechanism is conflicting. p85 sHER3 may compete for HRG binding to HER2/HER3 heterodimers or disrupt HER2/HER3 heterodimers.

The ECD of HER3 is extensively glycosylated (5). N-glycan deleted mutant of p85 sHER3^{N418Q} suppresses HRG signaling via HER3-containing heterodimers more effectively compared with the wild type, and the combination of p85 sHER3^{N418Q} with lapatinib synergistically suppresses the proliferation of BrCa cells (111). Furthermore, p85 sHER3^{N418Q} attenuates HGRβ-induced nuclear accumulation of transcription factors HIF-1α and Nrf2 in BrCa cells and suppresses HGRβ-induced cell migration (112). p85 sHER3^{N418Q} may therefore have therapeutic potential in the treatment of BrCa.

Serum HER3 proteins have been detected in patients with bladder cancer, prostate cancer and hepatocellular carcinoma (113,114), but to the best of our knowledge, there are no studies regarding serum HER3 in BrCa yet.

5. Soluble HER4 isoforms

The *HER4* gene was initially isolated from human breast MBA-MB-453 cancer cells in 1993 (115) and mapped to chromosome 2q33.3-34 (116). The *HER4* gene comprises 29 exons and encodes a 180 kDa protein. The ECD of HER4 has high sequence identity to that of HER3, whereas the kinase domain of HER4 is homologous to that of EGFR and HER2 (115). HER4 is widely expressed in normal human tissues and in cancer cells. The role of HER4 in human malignancies is ambiguous. Upregulation and downregulation of HER4 expression have been observed in 7-29 and 18-75% of BrCa cases, respectively, and numerous studies have reported simultaneous upregulation and downregulation of HER4 (116). Recently, Lucas *et al* (6) reviewed the mechanisms underlying HER4 signaling specificity and proposed that HER4 homodimers act as tumor

suppressors when they predominate over HER4 heterodimers, whereas HER4 heterodimers function as oncogenes when they predominate over HER4 homodimers.

HER4 transcript has two alternative splicing sites, located in the extracellular juxtamembrane (JM) region and the cytosolic C-terminus (Cyt), respectively. Alternative splicing of the JM region produces two isoforms that differ by the insertion of either 23 (JMa) or 13 (JMb) alternative aa residues (117). Alternative splicing of the Cyt region produces Cyt1 and Cyt2 isoforms, the latter of which lacks a 16 aa sequence that is part of a consensus binding site for the p85 subunit of PI3K (118,119). As a result, HER4 has four alternatively spliced isoforms: JMa/Cyt1, JMa/Cyt2, JMb/Cyt1 and JMb/Cyt2 (Fig. 1) (120). It is the HER4 JMa isoform, rather than the JMb isoform, that is susceptible to proteolytic cleavage (121-123).

Generation of HER4 ECD by proteolytic cleavage. PKC activators, such as phorbol ester and platelet-derived growth factor (122,123), and estradiol (121) can mediate the cleavage of HER4 JMa by ADAM17/TACE near the transmembrane domain (between H641 and S642), resulting in the shedding of a 120 kDa HER4 ECD (Fig. 2D) and the production of a membrane-anchored HER4 C-terminal fragment (4-CTF). Subsequent intramembrane cleavage of the 4-CTF by γ-secretase generates an 80 kDa soluble ICD of HER4 (HER4 ICD) (6).

Biological functions of HER4 ECD. It has been demonstrated that anti-HER4 mAb 1479 binds the extracellular subdomain IV of HER4 and inhibits HER4 cleavage. In a mouse T47D xenograft model, mAb 1479 significantly inhibits tumor growth compared with the control antibody and reduces HER4 ECD level (121), suggesting that HER4 ECD promotes tumor growth *in vivo*. Currently, knowledge of the biological functions of HER4 ECD is limited, whereas HER4 ICD attracts greater research interest than HER4 ECD (124).

Diagnostic and prognostic potential of HER4 ECD. HER4 ECD is detected in the serum of both patients with BrCa and healthy controls (121,125), and elevated serum HER4 ECD (≥40 ng/ml) is observed in 21% of patients with BrCa compared with 0% of healthy controls. However, HER4 ECD levels are not significantly associated with clinicopathological parameters such as ER status or histological grade of differentiation (121). More studies are needed to determine the clinical relevance of HER4 ECD in BrCa.

6. Conclusions

Soluble isoforms of each EGFR family member, produced by alternatively splicing and proteolytic cleavage, have been identified in BrCa cells and/or tissues. Notably, sHER2 and sHER3 have multiple alternatively spliced isoforms (Fig. 2). p110 EGFR ECD is shed by a secreted MMP, but its identity remains unknown (23). HER2 ECD is mainly shed by ADAM10 (58), and HER4 ECD is shed by ADAM17 (121-123). However, the cleavage sites of these receptors all fall within the conserved consensus motif P/GX_{5-7}P/G (126). Among the soluble receptors, sHER2 and sEGFR isoforms are more extensively studied than sHER3 and sHER4. In recent years, the emerging roles of sHER2 and sEGFR isoforms as non-invasive diagnostic biomarkers and promising therapeutic targets have attracted significant research interest.

p110 sEGFR and p110 EGFR ECD are generated by alternative splicing and proteolytic cleavage, respectively (7). Serum sEGFR levels vary greatly among patients with BrCa and do not show a consistent correlation with EGFR expression in BrCa or clinical outcomes. Although serum sEGFR levels lack reliable prognostic value, low levels of sEGFR and high levels of HER2 ECD in patients with mBrCa are associated with a poor prognosis (29); therefore, concurrent measurement of these two serum proteins may be more promising in the prognosis of BrCa.

Alternative splicing of HER2 produces multiple sHER2 isoforms, including herstatin, p100 HER2, HER2-I12 and Δ15HER2 (8). Shed HER2 ECD serves as a promising biomarker for HER2-positive BrCa. Elevated serum HER2 ECD predicts a poor prognosis, while its decline during therapy indicates a favorable treatment response. However, clinical use of serum HER2 ECD testing in patients with BrCa is limited by assay standardization, tumor heterogeneity, ethnic variability and interfering serum factors. Developing a multi-biomarker approach that includes HER2 ECD may improve the sensitivity and specificity of these tests, enhancing their prognostic value. In addition to HER2 ECD, CTCs, ctDNA and exosomal proteins are emerging biomarkers for early diagnosis and treatment of BrCa. Incorporating these liquid biopsy data into future studies may lead to a more precise and dynamic approach to BrCa diagnosis and treatment.

Herstatin is the most studied sHER2 isoform; it has been demonstrated to have therapeutic potential in HER2-positive BrCa; however, it has not yet been tested by clinical trials, probably due to difficulties in large-scale protein production, protein instability and challenges in targeted protein delivery. The approval of other HER2-targeted therapies in BrCa, such as trastuzumab and lapatinib, may potentially discourage the translation of herstatin into clinical applications.

sHER3 isoforms are produced via alternative splicing (109), but the clinical relevance of sHER3 in BrCa remains to be determined. HER4 ECD is generated by ADAM17-mediated cleavage of the HER4 JMa isoform (6). Limited evidence suggests a pro-tumorigenic role of HER4 ECD, but further research is needed.

EGFR signaling induces the immune evasion of TNBC cells (27), while HER2 modulates immune responses within the TME in HER2-positive BrCa (47). To the best of our knowledge, no publications have addressed the influence of soluble EGFR or HER2 on the tumor immune microenvironment. Investigating whether sEGFR family proteins modulate immune cells in the TME and uncovering the underlying mechanisms may serve to expand the treatment landscape for BrCa.

Taken together, soluble EGFR family members influence tumor growth, metastasis and treatment decisions. Advances in detection technologies, particularly nanotechnology-based approaches (29,78,79), will enable rapid, sensitive and cost-effective measurement of serum proteins. These technological improvements will be valuable for monitoring fluctuations in sEGFR and HER2 ECD during treatment, thereby facilitating treatment decisions. Integrative network analysis may help to identify prognostic biomarker panels

containing sEGFR family proteins and develop novel therapeutic strategies for BrCa (127,128). Further research is needed to understand the mechanism that regulates the release of sEGFR and HER2 ECD into the circulation and elucidate the diagnostic, prognostic and therapeutic potential of other soluble receptors in addition to sEGFR and HER2 ECD.

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Authors' contributions

PY drafted the manuscript; XJ prepared Figs. 1 and 2 and wrote and edited the manuscript; and FH proposed the outline, made comments, suggestions and revised the manuscript. All authors read and approved the final version of the manuscript. Data authentication is not applicable.

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Competing interests

The authors declare that they have no competing interests.

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