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Challenges in the clinical utility of the serum test for HER2 ECD

Lian Lam¹, Nicholas McAndrew¹, Marla Yee¹, Ting Fu¹, Julia C. Tchou², and Hongtao Zhang^{1,*}

¹Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

²Division of Surgical Oncology, Rena Rowan Breast Center, Abramson Cancer Center, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

Abstract

Approximately 15–30% of breast cancers over-express the HER2/neu receptor. Historically, over-expression of HER2/neu has been identified using IHC or FISH, both of which are invasive approaches requiring tissue samples. Recent evidence has shown that some tumors identified as “negative” using these methods can respond to HER2/neu targeted therapy. Shedding of the extracellular domain (ECD) of the receptor into the circulation has led to the development of serum test of HER2 ECD as an additional approach to probe HER2/neu overexpression. The serum test will be able to monitor the dynamic changes of HER2 status over the course of disease progression. Some studies further suggest that the serum HER2 ECD level and its change may serve as a biomarker to reflect patients’ response to therapy. Yet more than 10 years after the first serum HER2 ECD test was approved by the FDA, serum HER2 testing has yet to be widely used in clinical practice. In this article we will review the progress of the serum HER2 ECD test and discuss some obstacles impeding its incorporation into broad clinical practice. We will also discuss recent improvements in the sensitivity and specificity of the assay that offer some hope for the future of serum HER2 test.

Keywords

HER2; extracellular domain (ECD); targeted therapy; serum biomarker; detection

1. Introduction

The *HER2/neu* gene (also known as *c-erbB2*) expresses a 185 kDa transmembrane glycoprotein (HER2/neu, also known as p185^{her2/neu}) that is overexpressed in 15–30% of breast cancers [6] and other cancers [7, 8]. Her2/neu belongs to the ErbB family of receptor tyrosine kinases, which consists of four members: EGFR, HER2/neu, HER3, and HER4. Like the other members, the HER2/neu protein has three domains: a 105 kDa extracellular domain (ECD), a short transmembrane region, and an intracellular domain with tyrosine kinase activity.

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*Corresponding authors: Hongtao Zhang, Ph.D., 252 John Morgan Building, 3620 Hamilton Walk, Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104-6082, Phone: 215-573-9256, Fax: 215-898-2401, zhanghon@mail.med.upenn.edu.

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The importance of HER2/neu to tumorigenesis has been clearly recognized since the discovery of *neu*, the rat homologue of *HER2/neu*, as the oncogene that led to neuroblastoma and transformation of NIH3T3 fibroblast cells [10, 11]. In humans, breast cancers associated with *HER2/neu* amplification or receptor overexpression have an accelerated growth and recurrence-rate, and are associated with decreased overall patient survival [15].

1.1 HER2/neu as targets for therapies

Since HER2/neu is often amplified/overexpressed in cancer cells, it has been regarded as a tumor-specific target in molecular therapies. The original work of Drebin et al. using monoclonal antibodies targeting the onco-protein on tumor cells, has laid out the foundation for antibody therapies to HER2/neu as well as other tumor associated-antigens. Trastuzumab (Herceptin), the humanized anti-HER2/neu monoclonal antibody, was approved by the FDA in 1998 to treat metastatic breast cancer. The use of Trastuzumab in the treatment of operable HER2/neu positive breast cancer in the adjuvant setting began after the landmark study in 2005 {Romond, 2005 #437}. Later in 2010, trastuzumab was approved for metastatic gastric or gastroesophageal (GE) junction adenocarcinoma. Another anti-HER2/neu antibody, Pertuzumab, has completed clinical trials in combination with trastuzumab plus docetaxel on metastatic breast cancer and is currently pending FDA-approval. Lapatinib, a tyrosine kinase inhibitor with specificity for both EGFR and HER2/neu was also approved in 2010 to treat hormone receptor positive metastatic breast cancer. In addition, chimeric antigen receptor (CAR)-modified T cells was also developed as a cancer immunotherapy to target HER2/neu positive tumors [17], despite early indication of over-reactivity in clinical trials.

1.2 Diagnostic and screening tests for HER2/neu targeted therapies

For HER2/neu- targeted therapies, such as the trastuzumab-based therapy, a screening test for HER2/neu is required before treatment can be administered. Currently, HER2/Neu status is mainly evaluated using immunohistochemistry (IHC) and fluorescent in situ hybridization (FISH) (when IHC results are equivocal) in the clinical setting [21]. According to the most recent guidelines published by the American Society of Clinical Oncology and the College of American Pathologists, only patients with a uniform intense membrane staining of more than 30% of invasive tumor cells on IHC, and a *HER2/neu*-to-chromosome 17 centromere (CEP17) ratio of greater than 2.2 on FISH are considered HER2/neu-positive and are eligible for Trastuzumab treatment [22].

However, both IHC and FISH have been shown to have numerous limitations. One such limitation is the lack of ‘real-time’ follow-up due to the dependency of both assays on tumor biopsies. When patients diagnosed with HER2/neu positive breast cancer undergoing treatment return for follow-up, the Her2-neu receptor status of the treated breast cancer is not routinely re-tested. However, at times, the clinician may need to learn whether the HER2/neu status of the tumor has changed and to monitor treatment response. Neither IHC nor FISH is a practical assay. Inconsistent reporting of IHC or FISH may pose another problem which partly stems from variation in procedures by different labs and partly due to inherent heterogeneity in the distribution of HER2-neu positive tumor cells within the tumor. As many as 12% to 20% of the HER2/neu assays performed in the field may yield erroneous results due to intra- and interobserver variability, and non-standardized IHC assays and scoring systems [22–26]. In many cases there is also a significant discordance between the IHC and the FISH results. Loss of protein antigens in fixed tissue slides and subjective observations during the IHC procedure (IHC–, FISH+), as well as protein overexpression without gene amplification (IHC+, FISH–) account for such discrepancies. This raises many concerns as to whether IHC and FISH suffice for HER2/neu testing.

Serum extracellular domain fragment of HER2 (HER2 ECD) represents a noninvasive and quantifiable biomarker that could supplement existing HER2/neu testing. This extracellular fragment of the receptor is released from the surface of tumor cells into the circulation by the ADAM proteases (A Disintegrin And Metalloproteinase domain-containing protein) [30, 31]. Studies have found elevated levels of HER2 ECD in the serum of many breast cancer patients, and in most cases, high levels of serum HER2 ECD are associated with higher relapse rates and worse prognosis [29, 32]. However, the clinical usefulness of serum HER2 ECD has not been validated as a result of conflicting data. This review will focus mainly on the value of serum HER2 ECD as a biomarker for both diagnosis and prognosis, and recent developments in the field that could break through limitations of current serum HER2 ECD tests.

2. Prevalence of Her2 ECD in breast cancers

Many studies have measured the concentrations of serum HER2 ECD in breast cancer patients. We have reviewed 67 published studies (1991–2011) with prevalence of increased serum HER2 ECD in breast cancer patients (Fig. 1; Table S1). Of the 67 published studies, at least 5 different assays and 10 different cut off values were used. Some cutoff values were arbitrarily chosen based on serum values collected from healthy controls, whereas others followed the manufacturers' recommended cutoff value. Moreover, some of these studies used customized anti- HER2/neu antibodies for the assay.

Although most assays used the Immuno One (Bayer) selected cutoff value at 15 ng/ml it will be impossible to generalize a cutoff value as a cross-reference for all studies due to the variations between assays of these studies. In one study, abnormal HER2 ECD levels (2000U/ml) were found in 94% breast cancer serum samples but the selectivity/specificity was only 60%, indicating an unusual large percentage of healthy controls with "abnormal" levels above this cutoff [34]. Therefore, the cutoff level should be carefully defined to obtain the best sensitivity and specificity. In fact, Kong et al suggested that different ethnicities have different cut off values and it may be beneficial to establish cut off values for each ethnic population [35].

Nevertheless, the body of these studies has demonstrated that a great percentage (32% in average) of breast cancer patients have elevated serum HER2 ECD. Abnormal HER2 ECD is more frequently detected in patients after the disease progresses. Metastatic breast cancer (MBC) patients appear to have a higher percentage of positive serum HER2 ECD than primary breast cancer (PBC) (43% v.s. 18%) (Figure 1) [32, 37, 38]. Serum HER2 ECD prevalence rate from our analysis is in general consistent with previous report in 2003 [42].

3. Serum HER2 ECD and tumor tissue HER2/neu status

Determining the HER2/neu status is an integral part of the diagnostic workup for breast cancer patients. The precise status of HER2 is clinically important because it directs the course of therapeutic treatments for breast cancer patients. One original goal of serum HER2 ECD is to determine if this type of assay can be used to match the established FISH/IHC tests for HER2 status.

Many studies have evaluated the correlation between abnormal HER2 ECD in serum and HER2/neu positivity in tissue. Some of these studies have indicated a significant relationship while others have found no or poor correlations. Table 1 summarizes these studies.

In a prospective trial, Ludovini et al. found a concordance of 87.1% between elevated serum HER2 ECD levels (15 ng/ml) (23 patients, 9.0%) and HER2/neu-positive status in the tumor tissue (42 patients, 16.4%) of stage I–III breast cancer patients [19]. Correlation

between serum and tissue HER2/neu status was also observed in some other studies [3, 9, 14, 16, 20, 41]. Muller et al. reported a correlation between abnormal HER2 ECD and the tissue HER2/neu status in primary tumors of metastatic breast cancer patients ($p = 0.018$) [29].

Garoufali et al reported significant difference in serum HER2 ECD between early and advanced breast cancer ($p < 0.001$). However, a positive correlation of serum HER2 ECD with tissue HER2/neu status by IHC was only observed in advanced-disease patients ($p = 0.002$) [12]. In patients with distant metastases, a close correlation between expression of HER2/neu protein in the primary tumor and the serum HER2 ECD level was also reported by Narita et al. [39].

Correlation between serum HER2 ECD and tissue expression also extends to other tumors. In uterine serous carcinoma, patients with positive tissue HER2/neu were found to have higher serum HER2 ECD than patients with negative tissue HER2/neu or healthy female subjects [45].

However, several studies did not find a correlation between serum HER2 ECD and tissue HER2/neu status [1, 2, 40], especially in primary breast cancer without distant metastasis [44]. Sorensen et al. studied serum HER2 ECD in 1348 patients with breast cancer and found, in 206 HER2/neu positive patients, only 35 had abnormal serum HER2 ECD (16.9%) [46]. Of the 437 HER2/neu tissue negative patients, 69 were serum HER2 ECD positive (15.7%). Overall, these data demonstrate a moderate discordance between the tissue status and serum HER2 ECD levels in this assay.

It is important to note that the two HER2/neu tissue assays, IHC and FISH, are designed to examine HER2/neu at different levels: protein overexpression and gene amplification, respectively. As reported by Harris et al., serum HER2 ECD correlated reliably with tissue HER2/neu status by IHC but poorly with that by FISH [40]. However, current clinical practice favors FISH over IHC, as it is well perceived that FISH is more strongly correlated with responsiveness to either trastuzumab or lapatinib treatment [25].

The discrepancy between serum HER2 ECD levels and tissue status was somehow unexpected to researchers in the field and has become an obstacle for the clinical utility of the baseline HER2 ECD. There are several explanations accounting for this discrepancy:

Imperfection in the definition of tissue positivity for HER2/neu

One major source of discordance is the classification system for HER2/neu positivity. As mentioned before, to be qualified as HER2/neu positive, tumors have to contain more than 30% of positively stained tumor cells in IHC or show a HER2/CEP17 ratio of greater than 2.2 in FISH. It is argued that a population of patients may actually be incorrectly classified as HER2/neu negative because they had less than the recommended percentage of HER2/neu positive cells in the primary tumor and, therefore, did not fall under the category of HER2/neu positive [46, 47]. These HER2/neu positive cells in the primary tumor may have growth advantage and be sufficient to produce HER2/neu positive metastatic lesions. This may help explain why some HER2/neu “negative” patients can respond to trastuzumab. Likewise, the HER2/neu positive cells in the HER2 “negative” tumor can produce small amount of HER2/neu protein in the serum and become detectable with increased tumor volume.

Heterogeneity of breast cancers

There is some evidence that the origin and size of tumors can have a great influence on serum HER2 ECD levels. Pallud et al. found elevated serum HER2 ECD in 21 of the 157

primary breast cancers with paired serum and tissue samples [18]. HER2/neu tissue positive patients as determined by the Dako (3+) or CB11 (2+ and 3+) IHC had serum positive rate of 48% and 60% respectively. Most interestingly, abnormal HER2 ECD was observed in tissue positive patients only when the tumor histological size (pT) exceeded 28–30 mm. In all tissue positive patients with pure intraductal tumors (Dako+3), serum HER2 ECD was normal (<15ng/ml).

Conversion of HER2/neu status and the kinetic nature of serum HER2 ECD levels

Conventionally, IHC or FISH determines the classification of patients with HER2/neu positive or negative on the primary tumor that is generally removed a long time before therapeutic interventions. However, cancer is a progressive disease. Several publications have confirmed that the HER2/neu status of the primary and metastatic tumor can be discordant. Zidan et al. reported a 14% discordance in HER2/neu status between primary and its corresponding metastases from 58 patients [48]. The HER2/neu status changed from positive in the primary tumor to negative in the metastatic tumor in one patient, but 7 patients developed HER2/neu positive metastases from HER2/neu negative primary breast cancer. Hoefnagel et al also reported change in HER2/neu status in 5.2% of patients, with about half of them from negative to positive and the other half from positive to negative [49].

It is proposed that a small percentage of cancer cells with HER2/neu overexpression can exist in HER2/neu “negative” tumors. These HER2 shedding cells have growth advantage and their percentage could increase during the course of disease progression, leading to a transition of HER2 negative to positive tumor. [50]. Due to the progression of the disease state and the kinetic nature of serum HER2 ECD levels, it is highly possible that a patient could have both abnormal and normal serum HER2 ECD levels depending on the time of serum collection.

Asgeirsson et al studied paired serum samples collected at the time of primary and metastatic diagnoses from 57 patients [5]. Abnormal serum HER2 ECD (>15 ng/ml) was observed in 16 out of 57 patients (28%) at primary breast cancer diagnosis and in 31 out of 57 (54%) at metastasis. Serum levels changed in 18 patients from normal at the primary to abnormal at metastasis. More interestingly, serum levels changed in the opposite direction in 5 patients. In another study, the percentage of abnormal levels of serum HER2 ECD increased from 9.6% to 30.7% during the disease course. Only 9% of patients without recurrent disease had abnormal levels of serum HER2 ECD [51].

Surgery and treatment can also influence HER2 ECD serum levels. Fehm et al. retrospectively studied the kinetics of serum HER2 ECD levels for 52 primary breast cancer patients who had developed metastatic disease during follow-up [52]. Elevated baseline serum HER2 ECD levels were observed in 31% (16/52) of these patients. After the removal of the primary tumor, elevated serum HER2 ECD levels were still observed in 13% and 19% of patients at 3 months and 6 months, respectively. Nine out of 16 patients with abnormal serum HER2 ECD became normal, while 7 remained abnormal. Meanwhile, 3 out of the 36 patients with normal serum HER2 ECD developed abnormal levels by 6 months after surgery. At 6 months and 3 months prior to clinical diagnosis of metastases, elevated serum HER2 ECD levels were found in 27% and 50% of the patients, respectively. At metastases, 62% patients had elevated HER2 ECD levels. Twenty-four out of 45 metastatic patients had serum HER2 ECD changes inconsistent with response to first line treatment (hormonal or chemotherapy).

Definition of cutoff levels

Sorensen et al reported no correlation between serum HER2 ECD levels and tissue expression when using a cutoff value of 15 ng/ml. It is noted that the analysis included samples from patients who might have already been treated for breast cancer thus, HER2 ECD in those sample might already be reduced. Nevertheless, when compared to the actual ECD levels in tissue negative patients vs that in tissue positive patients, a significant difference can be detected ($p < 0.000001$) [46]. This raises the question if the cutoff value in this study was optimal.

In fact, Kong et al reported that a higher cutoff was required for serum HER2 ECD to correlate with tissue status [27]. In 195 patients with metastatic breast cancer, 76 (39%) were IHC 3+ and 19 were IHC 2+ but FISH positive. These patients have higher average serum HER2 ECD levels than HER2/neu tissue negative patients. ROC curve analysis was used to identify a cut-off level of 37 ng/ml with a specificity and sensitivity of 95% and 62%, respectively, in predicting tissue HER2/neu positivity.

In another study, Witzel et al found a correlation between serum HER2 ECD and tissue expression [41]. However, using 15 ng/ml as the cutoff only led to a sensitivity of 49%. ROC analysis suggested that better sensitivity (72%) could be obtained while good specificity (85%) retained when the cutoff was changed to 10 ng/ml.

Serum interference

A higher cutoff value means less impact of “noise” or false positive results in serum samples from some healthy controls and HER2/neu tissue negative patients. As we will discuss later in this review, serum interference might be a culprit for the unexpected elevated levels of serum HER2 ECD. Reported rates of elevated serum HER2 ECD among healthy controls vary significantly across studies, ranging from 4% to as high as 22.2% (Table 2). By reducing the serum interference, it is possible that HER2 ECD can better correlate with tissue HER2 status.

With the consideration of all these factors, it is unlikely that a strong consensus will be reached on the correlation between abnormal serum HER2 and tissue positivity, especially for early stage primary tumors. This lack of consensus stands as a hurdle for clinical adoption of serum HER2 ECD assays. However, increasing concerns have been raised surrounding the validity of tissue HER2/neu tests as the gold standard. It must be remembered that when using tissue HER2/neu status to select patients for treatment, only about 30% of patients respond to trastuzumab monotherapy [53].

The unexpected low response rate is not a clinical issue as the objective response rate is greatly improved when trastuzumab is combined with chemotherapies, which render FISH/IHC tests acceptable for the purpose of screening patients for targeted therapies. Nevertheless, recent discovery about benefit of trastuzumab based treatment for HER2/neu negative patients indicates that a better test is needed. With recent guidelines from the ASCO to increase the cutoff of tumor membrane staining from 10% to 30% [22], a larger subset of breast cancer patients may potentially be denied of early treatments by HER2 targeted therapies. Serum HER2 ECD has been studied to understand if it can have clinical utility as a helpful biomarker for therapies.

4. Clinical Utility of Serum HER2 ECD Measurement

Since the FDA approved the first HER2 ECD ELISA test in 2000, many studies have sought to define its clinical utility in reference to therapeutic response and other outcomes. These studies were first directed towards women with metastatic disease undergoing additional

therapy, but have progressed to include women with more limited disease burden. This section will provide an overview of the various clinical applications proposed for HER2 ECD assay.

In general, elevated serum HER2 ECD in metastatic breast cancer patients was found to link to much higher risk of disease progression [54], decreased disease-free survival and overall survival [55], and decreased survival-after-relapse [56]. Breast cancer patients with elevated HER2 ECD serum levels had a limited response to hormone or chemotherapy [13, 38, 40, 54] and a reduced response rate to tamoxifen (an antiestrogen) or letrozole (an aromatase inhibitor) [57].

A number of studies have monitored the circulating levels of ECD in response to HER2/neu targeted therapies, such as trastuzumab and lapatinib. Although some studies utilize these treatments as monotherapy, most include other chemotherapeutic regimens as well. In general these studies are designed to answer two questions: 1.) Is the baseline HER2 ECD level predictive of response to treatment? and 2.) Is the change of HER2 ECD levels from the baseline a better predictive biomarker of treatment response or disease progression?

4.1. Serum HER2 ECD Levels as a prognostic biomarker

A review by Carney et al based on 20 studies involving 4430 breast cancer patients indicates a strong correlation between abnormal HER2 ECD levels and worse prognosis (e.g. shorter time to progression (TTP), decreased overall survival (OS), and decreased progression-free survival (PFS) [42]. Metastatic breast cancer patients with abnormal HER2 ECD had a significantly worse OS and PFS than patients with normal ECD following high-dose chemotherapy and autologous stem cell transplantation [40, 58]. Pre-surgical high HER2 ECD in both node-negative and node-positive patients predicted a high possibility of metastases (mainly liver metastases) and poorer clinical outcome [33]. In one study, reduce OS (median: 17.1 months) was reported in metastatic breast cancer patients with abnormal ECD than those with ECD in the normal range [59].

In several studies, serum HER2 ECD was a better prognostic biomarker than tissue HER2/neu for metastasis and recurrence [40, 60]. Sorensen et al found 25 out of 35 tissue-positive and serum abnormal patients had recurrence in the form of metastases [46], suggesting a combination of serum and tissue HER2/neu status could be much more effective for prognosis.

4.2. Baseline serum HER2 ECD levels to predict response to HER2/neu Targeted Therapy

Several studies have reported no significant relationship between baseline serum HER2 ECD levels and clinical outcome in response to HER2/neu-targeted therapies [61, 62]. However, the combination of serum HER2 ECD testing with IHC and FISH can potentially identify, with more accuracy, patients who will more likely to benefit from trastuzumab treatment, and may provide useful information that is lacking in IHC and FISH testing, such as early diagnosis of recurrence and real-time biomarker for longitudinal assessments. Recently in a neoadjuvant therapy trial with breast cancer patients, Witzel et al found significantly higher pathological complete response (pCR) rate associated with abnormal HER2 ECD in HER2 tissue positive patients who received chemotherapies plus trastuzumab [41].

For metastatic breast cancer patients receiving trastuzumab (with or without other chemotherapy agents), Ali *et al.* showed there was a significantly better overall survival (OS) ($p = 0.017$) and time to progression (TTP) ($p = 0.007$) for patients who had normal baseline serum HER2 ECD levels (<15 ng/ml) than for patients with abnormal serum HER2

ECD [63]. This finding of reduced trastuzumab benefit in patients with abnormal HER2 ECD was unexpected.

One possible explanation for the reduced trastuzumab benefit may be linked to pharmacokinetic interferences with trastuzumab by HER2 ECD. In the study by Ali et al, the highest HER2 ECD baseline levels reached 4000–6000 ng/ml. Although the pharmacokinetic trough level of the therapeutic antibody is expected to be more than 10 µg/ml, the presence of extremely high concentrations of HER2 ECD (> 500 ng/ml) has been shown to greatly reduce the serum half life of trastuzumab from 9.1 days to 1.8 days, resulting in a very low trough trastuzumab level (<0.2 µg/ml) [64]. It has also been suggested that the presence of a large amount of circulating HER2 ECD might neutralize the therapeutic effect of trastuzumab. In cytotoxicity experiments, trastuzumab-mediated ADCC activity was significantly decreased by the addition of Her2 ECD [45]. These studies suggest that the pharmacokinetic interactions between trastuzumab and circulating levels of serum HER2 ECD must be considered and carefully evaluated in order to achieve optimal therapeutic effect on breast cancer patients.

In a study of the utility of HER2 ECD to predict response to lapatinib, 79% of tissue positive metastatic breast cancer patients had elevated HER2 ECD levels [65]. Out of these 138 subjects treated with lapatinib monotherapy, 33 patients had overall response (either complete or partial response). Patients with response to lapatinib monotherapy had higher baseline HER2 ECD levels than patients without response (mean 159.2 vs 94.1 ng/ml, $p = 0.043$) [65], but the baseline levels were not associated with progression free survival.

4.3. Change of serum HER2 ECD Levels for Monitoring HER2/neu Targeted Therapy

It turns out that the changes of HER2 ECD levels after HER2/neu-targeted therapy might be a better biomarker than the baseline itself. In one study, investigators found that a 9% drop in serum HER2 ECD between weeks 3 and 6 followed initial chemotherapy significantly predicted a pathological complete response (pCR) ($p = 0.04$) in 39 patients with local/locally advanced disease (stage II–III) who were treated with paclitaxel, fluorouracil, epirubicin and cyclophosphamide [61]. The pCR rate was also higher in the group receiving trastuzumab.

In another study, patients with a 20% or greater decrease in concentrations of serum HER2 ECD after receiving trastuzumab (with or without adjuvant chemotherapy) were significantly more likely to have a higher response rate, longer disease free interval (320 vs. 180 days) and increased overall survival (1023 vs. 503 days) than did patients who had a decrease in serum concentrations of 20% or lower [63]. Most importantly, in a subset of patients with HER2/neu tissue positive tumor, patients who achieved a significant decline in serum HER2 ECD had a much better clinical response than did patients without such decline: overall response rate: 58.3% vs 25%; median time to progression: 334 days vs 173 days; overall survival: 1023 days vs 519 days [63].

Lipton and colleagues also found similar benefits of this 20% reduction in serum HER2 ECD from the baseline [65]. This study looked at 138 patients with metastatic disease who were taking lapatinib monotherapy as first line HER2/neu directed treatment (i.e. these patients were trastuzumab naïve). Patients were randomized to either lapatinib at the dose of 1500 mg daily or 500 mg BID. Serum was collected every 4 weeks for a total of 16 weeks. The investigators found that a 20% decrease from baseline conferred a significantly greater overall response rates as well as increased progression free survival. Additionally, they found that the overall response rate was significantly worse in patients who had a 20% increase of serum HER2 ECD from the baseline.

In contrast, one study found that a reduction of over 55% from baseline serum HER2 ECD levels is necessary to predict a significant therapeutic response in patients [28]. Using a ROC curve with therapeutic response as the outcome, the investigators determined that a 55% reduction was the minimal number to produce an odds ratio significantly different from 1.0. In addition, the authors also claimed that patients with baseline serum levels over 15 ng/ml who achieved a reduction of serum HER2 ECD levels to under 15 mg/ml at week 12 of treatment had a greater likelihood of therapeutic response ($p = 0.005$) [28].

Other studies also reported a decline in serum HER2 ECD in responding patients [37, 41, 66]. Decline of serum HER2 ECD levels could be observed as early as 8 days after treatment started, and disease progression could be predicted by the decline of serum HER2 ECD at day 15 of treatment [66].

There have been reports, however, of responsive patients who experienced no reduction in serum HER2 ECD. Ali et al observed 28% of patients who responded to trastuzumab-based therapy did not achieve a 20% decline in HER2 ECD [63]. This indicates that patients should not be taken off trastuzumab therapies only because no significant reduction of serum HER2 ECD levels is observed. Instead, these patients should be considered as unlikely to be very responsive and can have other treatment options if available.

4.4. Rising of serum HER2 ECD before clinical evidence of recurrence

While decline of serum HER2 ECD may indicate response to therapies, rising levels may also foretell disease progression. As shown in Fig 1, up trend in the percentage of HER2 ECD abnormal patients can be seen from primary breast cancer to recurrent disease and to metastatic breast cancer. In a study by Fehm et al, abnormal ECD was found in 27% patients at 6 months before metastasis, 50% at 3 months before metastasis, and 62% at the time of clinically diagnosed metastasis [52].

In a study of 250 primary breast cancers with no evidence of residual disease (NED) after radical treatment (radical mastectomy or simple mastectomy and radiotherapy), 95 patients developed metastases during follow-up (up to 4 years). Rising serum HER2 ECD to abnormal levels were recorded in 28.4% of these patients before diagnosis of metastasis, with a lead time of 4.2 ± 2.4 months [67]. In patients with HER2/neu tissue positive primary tumors, rising HER2 ECD levels predicted 83.3% metastasis (10 out of 12 patients). Similarly, rising serum ECD levels was detected 3–24 months before recurrence was clinically confirmed in 10 HER2/neu tissue positive patients [46].

Schondorf et al monitored serum HER2 ECD changes in 23 patients with HER2/neu tissue positive tumor on trastuzuma therapy. All patients were treated by mastectomy and axillary lymph node dissection and all tissue histologically displayed the invasive ductal subtype [68]. Serum HER2 ECD were collected and measured periodically from these patients for circulating levels of HER2 ECD. To study the change of HER2 ECD levels, a total of 19 patients with HER2 ECD over 20 ng/ml were analyzed, and rising HER2 ECD levels were observed in 12 of these patients (63%). Within this group, 35 clinical remissions or progressions were documented, and changes of HER2 ECD levels correlated with 26 of these events (74%). Patients with very high HER2 ECD levels appeared to be associated with liver metastases.

Pectasides et al studied metastases after trastuzumab therapy [69] and found 37% of metastases were HER2/neu tissue negative, despite the primary tumors were positive. Five out of 16 patients had elevated serum HER2 ECD levels at baseline, and the levels returned into the normal range during treatment. However, 2 patients regained abnormal serum HER2 ECD levels and the metastasis tissue confirmed HER2/neu positive. Serum HER2 ECD

levels in the other three patients remained in the normal range and the metastasis tissue were HER2/neu negative. This study indicates that serum ECD might serve as a way to predict the status of metastasis. With more targeted therapy becoming clinically available for both HRE2 positive and negative tumors, the patients may be able to choose other treatment options without waiting for test from metastatic tissues.

4.5. Issues in monitoring HER2 ECD change

However, not all studies consistently confirm the correlation of reduction of serum HER2 ECD levels with response to treatments [70]. Lennon et al retrospectively analyzed data from four different clinical trials involving trastuzumab treatment on both metastatic and locally advanced breast cancer [62]. Serum HER2 ECD levels was determined using the commercial ELISA from Oncogene Science (Cambridge, MA). Although post-treatment reduction of serum HER2 ECD levels was observed in some patients with significantly elevated levels at baseline, there was no indication that the baseline level or the change of serum HER2 ECD could be used to predict treatment outcome.

Of note, the authors did not count changes within the normal range of serum HER levels (under 15 ng/ml) as significant. It is suggested that a defined percentage of changes should be used to analyze whether the response to treatment is linked to only significant changes of the serum HER2 ECD levels [71]. In a study by Lennon et al., a clear relationship between reduction of HER2 ECD and response was only noticed when trastuzumab was used as monotherapy [62]. It is not clear if chemotherapeutic agents that induce death of tumor cells can also release certain levels of the tumor receptor into blood, thus complicating the analysis of the change of HER2 ECD levels. It is also recommended that a prospective, not retrospective, study should be conducted to clarify the controversies in the field [72].

It has also been reported that HER2/neu targeted therapies can interfere with the release of HER2 ECD from tumor cells. Trastuzumab was shown to prevent the cleavage of ECD as it binds around the juxtamembrane cleavage site [73]. On the other hand, lapatinib was reported to increase the release of ECD [74].

4.6. Elevated serum HER2 ECD in patients with HER2/neu tissue negative breast cancer may predict response to trastuzumab based treatment

It has been documented that a certain percentage of primary breast cancers with negative tissue HER2/neu status develop abnormal HER2 ECD levels in serum [9, 16, 43]. Initially this observation was just considered as discrepancy between different methods assessing HER2 levels. It is now realized that this population might be responsive to HER2/neu targeted therapies. Thus, a potential utility of HER2 ECD is the identification of patients who miss the opportunity of being treated with HER2/neu targeted therapies due to lack of overexpression HER2/neu in tumor tissues.

Ardavanis et al. focused on women with metastatic disease who had negative tissue statue but abnormal ECD levels in serum [75]. These patients had all failed at least two regimens of chemotherapy, but none of them had received trastuzumab previously as they were FISH negative. Five out of 22 women who later received trastuzumab-based chemotherapy had a reduction in tumor mass and 11 other had stable disease after trastuzumab treatment. In this study, patients with the highest serum HER2 ECD levels (~ 250 ng/ml) at baseline were found to have reduced levels after 3 courses of treatment, accompanying tumor reduction in 4 patients and stable disease in 3. Considering the advanced disease stages and the resistance to previous therapies, the 73% beneficial effect (16/22) in this study strongly suggests that the HER2/neu tissue negative but serum positive patients should be investigated at a larger scale for trastuzumab benefit in this population.

4.7. Serum HER2 ECD Levels for Monitoring other Therapies

Although the previously mentioned studies have focused on monitoring serum HER2 ECD levels after HER2/neu-therapy, this biomarker is also useful in other non-targeted treatment. Decline of serum HER2 ECD levels from abnormal to normal was found in 9 out of 16 patients underwent surgery to remove tumors [52]. Reduction of serum HER2 ECD levels was also noticed in the control arm of patients receiving no trastuzumab but only chemotherapy treatment [62]. Breast conserving therapy offered after neoadjuvant therapy was reported to link to decline of HER2 ECD levels in HER2/neu tissue positive patients but not in tissue negative patients [41].

Muller *et al.* studied 103 women with metastatic breast cancer who were randomized to either epirubicin and paclitaxel or epirubicin and cyclophosphamide [29]. Of these patients, only 46 follow-up serum samples were taken. An increased serum HER2 ECD baseline (above 15 ng/mL) was associated with decreased overall survival, but not with shorter progression free survival or response to treatment. However, patients in the epirubicin and paclitaxel arm with increased initial serum HER2 ECD level had increased progression free survival compared to the other arm ($p = 0.0341$). In patients showing a complete response, their average serum HER2 ECD levels dropped from 17.1 to 11.4 ng/mL (or a 33% decrease) after 3 courses of therapy. This was not seen in patients with no change in clinical course or in patients with progressive disease.

Serum HER2 ECD was also studied to predict response to metronomic chemotherapy in patients with advanced breast cancer [76]. Abnormal serum HER2 ECD at 2 months after treatment, as well as an increase of HER2 ECD levels from baseline, was significantly associated with reduced long-term clinical benefit (24 weeks) ($P < .001$).

For patients with HER2/neu tissue negative primary tumors, monitoring serum HER2 ECD levels may identify patients with HER2/neu status conversion so they can benefit from HER2/neu targeted therapies. In one such study, Zidan *et al.* identified 7 HER2 ECD positive metastases in patients with negative primary tumors, four of which were offered trastuzumab-based therapy after chemotherapy failure, and three of them responded to trastuzumab [48]. In another study with HER2/neu negative primary breast cancer patients who received tamoxifen or letrozole, 26% converted from serum HER2 ECD negative to positive at the time of disease progression and showed significantly shorter survival compared to patients who remained negative for serum HER2 ECD [77].

5. Developments for future serum HER2 ECD test

Serum HER2 ECD concentrations are mostly assessed by ELISA based assays. Although ELISA is a relatively straightforward assay that is conventional in many labs, there are reports on incompatibility issues for some antibodies if the serum samples are from patients who are treated with trastuzumab. Hayashi *et al.* compared an EIA assay (Nichieri Biosciences) with CLIA assay (Bayer) and reported greatly reduced readout with the EIA assay in patients receiving trastuzumab treatment [70]. It was suspected that trastuzumab from treatment remained in the circulation and might compete with the two antibodies used in the EIA assay (6G10 and SV-2-61). In some other assays, such as the Siemens Immuno-1 automated assay, trastuzumab was tested not to interfere with the assay [78].

Even with optimized antibody pairs for ELISA, testing HER2 ECD from serum or plasma still faces some issues that are introduced by clinical samples. Emerging technologies over the past two decades have enhanced or completely revolutionized existing immunological tests. We have seen some efforts in the field to improve the sensitivity and specificity of the

assay, which may help to develop the next generation of serum tests to help HER2/neu targeted therapies and other treatments.

5.1. Emerging Assays with higher sensitivity

While conventional methodologies such as ELISA and EIA are still widely used, they are limited by the concentration of the antigens and their sensitivity is generally in the ng/ml range (24, 101). Since the normal serum HER2 ECD level (< 15 ng/ml) is very close to the limit of ELISA assay, the conventional HER2 ECD ELISA assays are susceptible to assay interference that produces noise and false results. The development of chemiluminescence immunoassays (CLIA) has improved the sensitivity and its usefulness has been confirmed in serum HER2 ECD of breast cancer patients [37, 70]. Detection limit, however, is still an issue and highlights the need for ultrasensitive immunological tests. Loo et al developed a piezoelectric microcantilever sensors (PEMS) with 5 fold higher sensitivity than ELISA and was able to detect HER2 ECD in serum at concentrations below the low end of normal range (2 ng/ml)[79]. A new assay based on linear RNA amplification termed Fluorescent Amplification Catalyzed by T7 RNA polymerase Technology (FACTT) reported even higher sensitivities [80].

In FACTT, the antigen of interest is captured by the plate-associated antibody and detected by antibodies coupled to a double stranded DNA template that accommodates the attachment of the T7 RNA polymerase enzyme. The interaction of T7 leads to the production of RNA species that is monitored with a fluorescent RNA intercalating dye (RiboGreen, Invitrogen). We have applied FACTT to multiple protein targets, including HER2 ECD, TNF α , G-CSF, and the prion protein, and demonstrated at least 1000-fold higher sensitivity than ELISA [81]. In addition, FACTT is not subjected to background noise that is associated with substrate in ELISA assays. In a study of ten women with FISH+ tumors, FACTT detected elevated HER2 ECD in eight, while ELISA only detected elevated levels in two [80].

5.2. Approaches to enhance selectivity in serum test

One persistent problem that plagues serum biomarker tests by ELISA is the false results caused by the heterophilic human anti-animal immunoglobulin antibodies (HAIA), including human auto-antibodies that bind to animal antibodies by cross reactivity [82, 83]. An example of HAIA is the human anti-mouse antibodies (HAMA), which are generated in people who have come in contact with mouse immunoglobulins or present as human auto-antibodies but with cross reactivity.

HAIA are easily acquired through blood transfusions, vaccinations, treatment with mouse monoclonal antibodies, or from handling animals [16]. The prevalence of HAIA is varied in different reports, ranging from $<1\%$ to 99% of the population and from $\mu\text{g/mL}$ to mg/mL concentrations in serum [16]. In one study, human anti-bovine antibodies were detected in 99% of the donor serum samples [84].

HAIA or HAMA is known to interfere with a number of immunological assays used for detecting cancer biomarkers, including serum HER2 ECD. It has also been reported to interfere with ELISA tests used for measuring IL-8, prostate specific antigen, and serum thyroglobulin levels in melanoma, prostate, and thyroid cancers, respectively [85–87]. In ELISA assays, HAMA can crosslink the capture and signal antibodies giving a false positive result, or it can bind to the capture antibody, sterically blocking binding of the signal antibody, resulting in a false negative reading [85, 88, 89].

Misleading results often result in inappropriate or unnecessary therapies, evident from the nationally publicized Rufer case. Rufer was diagnosed to have a form of cancer in her uterus

by a human chorionic gonadotropin (hCG) blood test. The test stayed positive after she underwent chemotherapy and later hysterectomy. In fact, the test was repeated 44 times with the assay made by Abbott Laboratory, all showing abnormal levels of hCG. More chemotherapy was administered and a full-body CT-scan identified a suspicious nodule in her lung. As a result, a portion of her lung was removed to prevent further “metastasis”. Eventually, an hCG test manufactured by a different company was performed and the result was negative. It turned out that the previous results were all false positives.

To avoid HAIA-related problems in ELISA assays, many approaches have been reported to reduce interference, including removing immunoglobulin from serum samples [90], using mouse IgG-derived blocking reagents [91], and replacing antibody with non-IgG detection agents [92]. A unique approach was developed to resolve this issue using a universal blocking buffer to dilute serum samples [86]. In the ELISA assay, non-specific signals caused by HAIA mediated serum interference can be noticed when a positive reading is recorded with the use of non-matching pair of antibodies that recognize different unrelated proteins. A specially designed MBB buffer was shown to eliminate such an interaction [86].

To visualize the potential for serum interference in a serum HER2 ECD test, we performed an ELISA with recombinant HER2 ECD serially diluted in PBS, a standard buffer used in ELISA assays, as the standard curve. In parallel, the same dilution was performed but each sample was spiked with a HAIA serum sample (at 1:20 dilution). As shown in Fig 2, the calculated concentrations of HER2 ECD in the spiked samples were statistically different from the expected concentrations (paired *t* test, $p = 0.012$). A background reading (20–30 ng/ml) appeared when there was no or very low concentration of recombinant HER2 ECD. On the other hand, a very high reading of 162.9 ng/ml was detected when the sample only contained 50 ng/ml recombinant protein. In contrast, when the ELISA was performed with the MBB buffer, a close correlation between the detected and expected concentration in samples containing HAIA was observed ($p = 0.975$).

Considering the lack of information on the serum interference issue in a considerable numbers of previous studies, it is highly possible that some HER2 ECD levels in the literature might be misleading. The issue of HAIA could be significant if results of these studies were based on a single pair of antibodies and only a very small number of patients were surveyed. However, as most of these studies involved a sufficient number of subjects, the impact of serum influence is minimized. Nevertheless, it is highly recommended that future HER2 ECD tests should include HAIA-preventing mechanism such as the MBB buffer.

Summary

Prevalence of elevated serum HER2 ECD in breast cancer patients has been documented in many studies. The abnormal serum HER2 ECD levels are more frequently observed in metastatic breast cancers. The concentration of HER2 ECD in serum is dynamic during disease progression and treatment. Baseline levels may not correlate with tissue status, especially in early stage breast cancer patients, while serum HER2 ECD in metastatic breast cancer is more closely correlated with tissue expression of HER2/neu proteins. Monitoring of HER2 ECD will not replace FISH/IHC assays but definitely will complement the tissue assay to offer a real time picture of HER2/neu status in patients. Decline of serum HER2 ECD levels after treatment (>20%) might indicate response to treatments. While other assays exist that may offer increased sensitivity over ELISA in measuring serum HER2 ECD, there are also methods (MBB buffer) that may further refine the specificity of ELISA testing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Prevalence of serum HER2 ECD in Breast Cancer

Figure 1. Prevalence of serum HER2 ECD in breast cancer

This is a summary of 70 studies published between 1991–2011 (see Table S1 for detail). In average, abnormal levels of HER2 ECD were found in 33% of all breast cancers (Total BC), 18% of primary breast cancer (PBC), 23% of breast cancers with locoregional recurrence (loco Recur), and 44% of metastatic breast cancer (MBC).

Figure 2. Detection of recombinant HER2 ECD in samples containing HAIA serum
Recombinant HER2 ECD (eBioscience, Inc.) at different concentrations but with consistent levels of HAIA serum (5%) was detected using anti HER2/neu monoclonal antibodies 6E2 and biotin-A21. The presence of MBB buffer corrected the influence of the HAIA serum and showed good correlation between detected and expected HER2 ECD levels.

Table 1

Summary of studies for the relationship between abnormal HER2 ECD in serum and tissue expression positivity.

Reference	Patient population	Cutoff	Serum Assay	Correlation?
Cheung et al [3]	20 MBC	20 ng/ml	Bayer Immuno1	Yes.
Anderson et al [9]	168 PBC (*)	1600 HNU/ml	Oncogene Science	Yes.
Garoufali et al [12]	116 MBC	12.7 ng/ml	Bayer Immuno1	Yes.
Colomer et al [13]	40 MBC	450 fmol.ml	Calbiochem	Yes.
Sugano et al [14]	158 PBC	5.4 ng/ml	Nichirei	Yes.
Krainer et al [16]	47 PBC	20 U/ml	Triton Diagnostic	Yes.
Pallud et al [18]	157 PBC	15 ng/ml	Bayer Immuno1	Yes for invasive
Ludovini et al [19]	256 PBC	15 ng/ml	Oncogene Science & ADVIA Centaur	Yes.
Farzadnia [20]	74 PBC	18.4 ng/ml	Bender MedSystem	Yes
Kong et al [27]	195 MBC	37 ng/ml	ADVIA Centaur System	Yes
Fornier et al [28]	55 MBC	15ng/ml	Bayer Immuno1	Yes.
Muller et al [29]	29 MBC	15 ng/ml	Oncogene Science	Yes.
Molina et al [33]	77 MBC, 84 locoregional	15 U/ml	Ciba Corning	Yes
James et al [36]	100 MBC	15 ng/ml	Bender MedSystem	Yes
Narita et al [39]	55 PBC	20 U/ml	Triton Diagnostic	Yes for MBC, no for PBC.
Harris et al [40]	355 MBC	20 U/ml	Chiron Diagnostics	Yes
Witzel et al [41]	167 PBC	15 ng/ml	Siemens	Yes
Kandl et al [43]	24 MBC	10 U/ml	Triton Diagnostic	No.
Willsher et al [2]	57 PBC & MBC	20 ng/ml	Bender MedSystem	No.
Quaranta et al [1]	108 PBC	1368 HNU/ml	Oncogene Science	No.
Fontana et al [44]	62 PBC	8 U/ml	Triton Diagnostic	No.
Kong et al [35]	86 PBC	10.2 ug/L	ADVIA Centaur System	No.
Sorensen et al [46]	826 MBC	15 ng/ml	ADVIA Centaur System	No.

Table 2

Abnormal serum HER2 ECD in healthy controls.

Authors (reference)	Total number of healthy patients	Abnormal HER2 ECD (%)
Quaranta et al [1]	63	22.2
Willsher et al [2]	23	13
Breuer et al [4]	25	4
Asgeirsson et al [5]	49	20.4