Original Article

Serum Human Epidermal Growth Factor Receptor-2 (sHER-2/neu) in breast cancer patients and its comparison with clinicopathological parameters

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Abstract:

Background- Breast cancer patients with HER-2/neu amplification or overexpression are eligible for treatment with transtuzumab (Herceptin). In clinical practice, over-expression of HER2/neu is routinely identified using Immunohistochemistry (IHC) and Fluorescence in situ Hybridization (FISH), both of which are invasive approaches requiring tissue samples.

The sECD/HER-2/neu (Extracellular domain) fragment from the surface of breast cancer cells once shed into the blood of individuals can be quantified using commercially available enzyme-linked immunosorbent assays, making it a useful breast cancer biomarker.

Objective- To compare the levels of sHER2/neu with IHC and find the association of sHER2/neu with clinicopathological parameters.

Material and methods- 75 histologically confirmed female breast cancer patients in the age group of 18-80 years were recruited. sHER2/neu levels were measured by RayBio Human ErbB2 Elisa kit. Cut-off value of >15ng/ml was used to define elevated sHER2/neu.

Results- 70.6% (53/75) of the patients were sHER2/neu positive and 53.3% (40/75) were IHC positive. There was a significant correlation found between sHER2/neu and IHC (p=0.05). Statistically significant association was found between sHER2/neu and lymph node status (p= 0.004), histological grade (p=0.006) and clinical stage (p=0.015). No statistically significant association was found between sHER2/neu and age, menopausal status, histological tumor type, Estrogen and Progesterone receptor (ER, PR) status and molecular type.

Conclusion- sHER2/neu by ELISA will definitively complement the tissue assays to offer a real time picture of HER2/neu status. sHER2/neu testing may be a useful tumor marker for monitoring breast cancer patients, even in those with negative IHC.

Keywords: sHER2/neu, Elisa, Tumor marker, breast cancer

INTRODUCTION:

An estimated 1.38 million women worldwide are diagnosed annually with breast cancer (1). A study of breast cancer in India revealed that 1 in 28 women develop breast cancer during her lifetime (1 in 22 in urban areas). There is a significant need and interest to identify prognostic and predictive factors of the disease to help us understand its natural history and guide on therapeutic selection (2).

Human Epidermal Growth Factor Receptor-2 (HER-2/neu or c-erb-B2) gene is a protooncogene mapped on chromosome 17q. It encodes a transmembrane tyrosine kinase growth factor receptor that is expressed on cells of epithelial origin. In Indian women with breast cancer the HER2/neu gene is amplified in 30% of cases (3).

The full-length glycoprotein (p185HER2) has a molecular mass of 185 kDa and is composed of an internal tyrosine kinase domain, a short transmembrane portion, and an extracellular domain (ECD) . The ECD of the receptor protein is heavily glycosylated and has a molecular mass 97-115 kDa. Further, this full-length glycoprotein receptor undergoes cleavage by ADAM proteases (A Disintegrin and Metalloprotein domain containing protein) resulting in the release of the soluble shed ECD (sECD-HER-2/neu) fragment. The resultant truncated intracellular form containing the kinase domain is associated with enhanced signaling activity and consequently contributes to metastatic breast cancer.Binding of peptide ligands (Hergulins) result in dimerisation, transphosphorylation of tyrosine residues, resulting in cell growth and differentiation through signaling cascades (4)

Aggressive breast tumors showing resistance to cytotoxic and endocrine therapies have been found to be HER-2/neu positive. Patients with HER-2/neu amplification or overexpression are eligible for treatment with transtuzumab (Herceptin), a fully humanized monoclonal antibody directed against HER2. The antibody seems to cause internalization and degradation of the ECD, inhibiting the signal transduction pathway.

In routine practice, HER-2/neu tissue status is assessed using two methods; immunohistochemistry (IHC) which measures over-expression of the HER-2/neu full-length oncoprotein (p185); and fluorescent in situ hybridization (FISH), which measures the number of HER-2/neu gene copies. A third tissue method is known as chromogenic in situ hybridization (CISH), but is not yet approved by FDA. These three tests are performed on breast tissue that has been formalin fixed and embedded in paraffin, and findings are reviewed to determine HER-2/neu status. The American society of Clinical Oncology and the College of American Pathologists guidelines states that only patients with a uniform intense membrane staining of more than 30% of invasive tumor cells on IHC, and a HER-2/neu- to-CEP 17 ratio of greater than 2.2 on FISH are considered HER-2/neu positive and are eligible for transtuzumab treatment (5).

Laboratories nowadays employing IHC generally use either Herceptest or CB11 to measure the level of p185 expression. However, both IHC and FISH methods are laborious, demand a high skilled eₓpert and 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 HER-2/neu status of the tumor has changed and to monitor treatment response. Neither IHC nor FISH is a practical assay. Studies collectively show that the HER-2/neu status of the primary breast cancer (PBC) does not always accurately reflect the HER-2/neu status of metastatic breast cancer (MBC). Over the past few years numerous reports have been

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published identifying discrepancies between Herceptest and CB11, between IHC and FISH, and between FISH and CISH, as well as discrepancies between various IHC and FISH methods from different manufacturers. Because all three tests (IHC, FISH, CISH) use fixed tumor tissue, there are several sources of potential error. For instance, the HER-2/neu epitope can be destroyed by formalin fixation and many years of storage. Several other conditions can contribute to false-positive or false-negative IHC results. As many as 12-20% of HER-2/neu assays performed in the field may yield erroneous results due to intra- and inter observer variability in tissue processing, scoring interpretation, reagent variability, antigen retrieval methods, tumor heterogeneity, and the semiquantitative nature of the test (6).

A current, rapid method to detect HER-2/neu protein in biological fluids, such as serum, is the Enzyme-Linked Immunosorbant Assay (ELISA). A typical commercial HER-2/neu ELISA uses the "sandwich" principle, where a capture antibody is directly adsorbed onto a substrate. The detector antibody is labeled with an enzyme, which upon addition of the substrate, produces a colored product quantifiable by absorbance analysis (4). The ECD of HER-2/neu is cleaved and released into peripheral circulation (sHER-2/neu ECD) making it a logical prognostic and/or predictive marker in metastatic and early stage HER-2/neu expressing breast cancer (2).

The serum HER-2/neu test cleared by the FDA is intended to measure HER-2/neu ECD quantitatively in serum of women with MBC. Many studies have shown that increases and decreases in serum HER-2/neu levels correlate with the clinical course of disease when monitored in serial samples of MBC patients (7). A significant association was observed between serum HER-2/neu ECD levels and expression of HER-2/neu in primary tumors, with concordance indices for HER-2/neu of 80% (4). Nevertheless, there is limited information regarding the use of circulating HER-2/neu to predict benefit from transtuzumab treatment in PBC (8).

ELISA is a reliable and economical tool to assess the HER-2 status in tumors, when breast tissue sample is not available (9). The advantages of this method are that it is easy, reproducible, quantitative, and objective and blood is routinely collected in the physician's office before administration of chemotherapy. Moreover, blood collection provides a real-time analysis of the patient's serum HER-2/neu status at the time clinical decisions are made concerning therapy. High sHER-2/neu ECD have been associated with tissue HER-2 over-expression (10), increased tumor burden (11), poorer survival (12), and resistance to endocrinotherapy and chemotherapy (13). Studies have found that HER-2/neu ECD positivity was significantly associated with most clinicopathological parameters including tumor size, lymph node involvement, tumor stage, histological grade, chromosome ploidy, ER, PR, and HER-2/neu membrane protein over-expression (14).

MATERIAL AND METHODS:

After the Ethical clearance, 75 histologically confirmed female patients in the age group 18-80 years attending the Cancer Research Institute, Dehradun were included. Histologically benign breast tumors, previously treated breast cancer cases or patients suffering from other types of cancer as well, or those with incomplete files were excluded from the study. Information on patient's age, menopausal status, disease stage, grade, tissue HER2/neu status, estrogen receptor (ER), progesterone receptor (PR) status, clinical nodes were noted from the case files. Those samples who were tissue HER2/neu 2+ were further sent for FISH amplification test.

Serum HER2/neu levels were measured by modified sandwich enzyme immunoassay. The manufacturer's instructions on the kit (RayBio Human ErbB2 Elisa kit, Cat# erbB2-001) were followed. Structured study instruments (case study pro forma) were developed & used to generate data. According to the previous studies and FDA recommendations,15 ng/ml was the cut off for sHER2neu levels.

RESULTS:

Mean age of the cases were 47.97 +- 12.67 years (35.3-60.6 years). Median age was 45.5 years. 71 (95%) are IDC (Invasive Ductal Carcinoma) type, 2 (3%) ILC (Invasive Lobular Carcinoma), and 1 each of Tubular and Comedo type ; mean sHER2/neu values were 17.98 ng/ml, 24.2 ng/ml, 21.7 ng/ml, 16 ng/ml, respectively.

35 (44%) of the patients were tissue HER2/neu negative (0/1+) and 45 (56%) patients were tissue HER2/neu positive (3+/ 2+, FISH amplified). 22(29%) patients had sHER2/neu <=15 ng/ml and 53 (71%) patients had sHER2/neu >15 ng/ml. Mean serum value is 18.26 (+- 7.5) ng/ml. 28 (37%) of the patients were under 40 years of age and 47 (63%) of the patients were above 40 years of age. The mean sHER2neu values of the <40 years and >40 years age groups were 20.5 and 19.5 ng/ml respectively. There was no statistical difference between the mean sHER2/neu values of the patients two age groups. 37 (49%) are premenopausal and 38 (51%) are postmenopausal women. The mean sHER2/neu levels in premenopausal group was 17.74 ng/ml and in the post menopausal group was 18.59 ng/ml. There was no statistical difference between the sHER2/neu levels of the pre-menopausal or post-menopausal patients.

(Table 1) 48(64%) patients are node positive and 27(36%) patients are node negative with mean sHER2/neu values being 19.7 ng/ml and 15.42 ng/ml, respectively. The comparison between the sHER2/neu value and the axillary lymph node status is statistically highly significant ($p<0.01$).

8 (11%), 28 (37%) , 36 (48%) and 3(4%) patients fall into clinical stages I, II, III, and IV with their mean sHER2/neu levels being 15.65 ng/ml, 16.01 ng/ml, 20.92 ng/ml, and 12.07 ng/ml respectively. The difference in sHER2/neu levels in various stages is statistically significant ($p<0.05$) (Table 2)

3 (4%) patients are in Grade I, 53 (71%) in Grade II and 19 (25%) in Grade III at the time of presentation. The mean sHER2/neu levels are Grades I, II, III are 23.70 ng/ml, 17.25 ng/ml, and 21.65 ng/ml respectively. The difference in sHER2neu levels in the 3 Grades is statistically highly significant ($p<0.01$) (Table 3)

All the molecular types were classified as serum negative (sHER2/neu <=15 ng/ml) and positive (sHER2/neu >15 ng/ml. Mean sHER2/neu levels in Luminal type A, Luminal type B, HER2/neu type, and Triple negative types in the negative and positive categories were 8.10 (SD 1.64) ng/ml, 20.51 (SD 3.09) ng/ml, 9.98 (SD 2.48) ng/ml, 24.07 (SD 7.43) ng/ml, 9.95 (SD 1.34) ng/ml, 21.3 (SD 2.75) ng/ml, 8.3 (SD 2.04) ng/ml, 20.47 (SD 1.91) ng/ml, respectively. The difference between sHER2/neu levels with the molecular types were not statistically significant ($p=0.202$).

Table 4 shows that 14 patients negative with sHER2/neu (<=15 ng/ml) were also negative on the tissue examination with IHC (0/1+). 32 patients positive with sHER2/neu were also positive for tissue HER2/neu (2+/3+). The equivalence between sHER2/neu with that of tissue was 0.75 (p=0.05) which was significant. No significant association was seen between sHER2/neu and ER, PR.

Multivariate logistic regression analysis showed an inverse risk relation between menopausal status and estrogen receptor status. By the adjusted OR (odds ratio) it was observed that the age>40 years, clinical stages I, II, III, and PR were positively related to the elevated HER-2/neu levels and menopause and ER were negatively related to the elevated sHER-2/neu levels (Table 5)

DISCUSSION:

In this study, elevated sHER2/neu were observed in 70.6% (53/75) of the breast cancer patients. This is higher than usual because we have included only those breast cancer patients for whom breast carcinoma panel (ER, PR, HER2/neu) was requested by the clinician. The usual prevalence of HER2/neu gene amplification is around 30% (8). But studies have shown that the percentage of elevated sHER2/neu ECD are extremely variable in early breast cancer patients at the time of diagnosis. Possible explanations for this are normal elevation of serum HER2 ECD level as reported in healthy controls (16), a minority of HER2-positive cells being lower than the definition of tissue HER2 positivity as its source (17), genetic differences between individuals with respect to matrix metalloproteinase activity, which is responsible for HER2 release into the serum, and chance false positivity (18). Patients with liver dysfunction have also reported abnormal sHER2/neu levels (19). Since patients were not followed for occurrence of metastasis or other clinical outcomes, the clinical implication of unexpected sHER2/neu ECD could not be further investigated.

Use of different cutoff values, small samples, and different patient populations in the various studies may have attributed to controversial association between sHER2/neu and tissue HER2 status.

Using a lower cutoff value could yield a higher sensitivity and negative predictive value, but it could negatively affect the specificity and positive predictive value. So, in view of the adjunctive role of serum HER2 ECD level, the cutoff of 15 ng/ml may have greater clinical utility. This is because higher specificity and positive predictive values are essential to reduce the likelihood of false suspicion of tissue HER2 status due to the abnormal serum HER2 ECD level.

In our study, 70.6% (53/75) of patients were sHER2/neu positive and 53.3% (40/75) were IHC positive. There was a significant correlation found between the two parameters (p=0.05). Many researchers using the same cut-off have suggested significant correlations between the expression of tissue HER2 and serum HER2 ECD level exist in primary breast tumor, regardless of whether the primary tumor is early or advanced (3,15,20,21,22,23). A significant association was observed between sHER2/neu and lymph node status (p=0.004), clinical stage (p=0.015) and histopathological grade (p=0.006), and not with age or menopausal status. Multivariate logistic regression analysis showed an inverse risk relationship between serum HER-2/neu levels and ER and PR status.

Earlier, V.Ludovini et al showed a significant association between sHER2/neu ECD with histological grade (P = 0.003), stage III (P = 0.008), lymph node involvement (P = 0.035), and a negative association with both estrogen (P = 0.016) and progesterone (P = 0.007) receptors. They said they could not find a statistical relationship between sHER2/neu levels and the other variables (age, menopausal status, histological tumor type and tumor size) (21).

Krainer M et al analyzed serum HER-2/neu primary breast cancer patients to find a significant correlation betweensHER-2/neu and tumor size (p < 0.0001) or axillary lymph node involvement (p < 0.0001).They observed that a small subgroup of breast cancer patients with a relatively advanced stage of disease can be diagnosed by

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measuring sHER2/neu levels. They showed that sHER2/neu has a strong correlation with tumor load in stage II and the high prevalence in stage IV disease, making it a good marker for the assessment of disease activity and biologic behavior in breast cancer (24).

High sHER2/neu levels were significantly associated with lymph node involvement (p=0.037) and ER negativity (p=0.017) as observed by Dong WR et al. No significant relationship was found between sHER2/neu levels and other variables, such as age, tumor size, PR, and p53 (25).

Farzadnia m et al did a cross-sectional study on 75 patients with breast carcinoma. There was a high statistical correlation between sHER2/neu levels and IHC (p=0.018).They found no significant correlation between sHER2/neu and age, tumor size, stage, grade and metastatic lymph nodes (26).

Rajeev Singhai et al showed that HER-2/neu levels were significantly related to age, clinical stage of disease, nodes, and hormone receptors including ER and PR, but not to menopausal status (3).

In our study 7/10 (70%) patients found TNBC on IHC had elevated sHER2/neu. This is an important finding since TNBC patients are usually considered resistant to targeted therapies and thus have bad prognosis.

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 (27). 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. Our 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.

CONCLUSION:

sHER2/neu testing may be a useful tumor marker for monitoring breast cancer patients, even in those with negative IHC for the HER2 protein. Its strong correlation with tumor load (lymph node metastasis, staging, grading) could make it a promising tool for assessment of disease activity, predicting response to therapy and early recurrence or metastasis. We strongly advocate that more studies with larger sample size should be done to assess its role in TNBC patients. The possibility of obtaining information simply with serum HER-2/neu determinations offers an attractive approach that is easy to implement, of low cost, and providing early and relevant information, especially in a developing country like ours.

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Table 1: **Comparison between sHER2/neu and axillary lymph node status**

Variable	Category	N(75)	sHER2/neu Median	sHER2/neu Mean	SD	p value
Node status	Positive	48	20.4	19.7	7.14	0.004
	Negative	27	16.12	15.42	6.14	

Table 2: **Comparison between sHER2/neu and clinical stages**

Clinical Stage		N(75)	sHER2/neu Minimum	sHER2/neu Maximum	sHER2/neu Median	sHER2/neu Mean	SD	p value
	I	8	8.00	23.70	16.2	15.65	5.83	
	II	28	6.00	29.70	17.2	16.01	6.77	0.015
	III	36	7.20	50.00	21.6	20.92	7.59	
	IV	3	6.70	21.00	8.5	12.07	7.79	

Table 3: **Comparison between sHER2/neu and histopathological grades**

		N(75)	sHER2/neu Minimum	sHER2/neu Maximum	sHER2/neu Median	sHER2/neu Mean	SD	p- value
Histopathological Grade	I	3	20.50	29.70	16.2	23.70	5.20	
	II	53	6.00	28.20	17.25	16.60	6.72	0.006
	III	19	6.70	50.00	21.65	21.64	8.59	

Table 4 : Comparison between sHER2/neu and Tissue HER2/neu (IHC) , ER, PR values

		Serum HER2/neu			
Tissue HER2/neu (IHC)		Negative(<=15ng/ml)	Positive(>15ng/ml)	P -value	Kappa
	NEGATIVE(35)	14 (63.63)	21 (39.62)		
	POSTIVE(40)	8 (36.36)	32 (60.37)	0.05	0.75
ER	NEGATIVE(27)	6(22.8)	21(77.8)	0.228	
	POSITIVE(48)	16(33.3)	32(66.7)		
	NEGATIVE(50)	19 (76)	6(24)		
PR	POSITIVE(25)	34 (68)	16(32)	0.331	

Table 5: Multivariate logistic regression analysis for predicting HER-2/neu levels in relation to age (>40 years), menopausal status, stages, ER(+), PR(+)

	B	S.E.	Wald	Sig.	Adjusted odd
AGE	0.988	1.040	0.902	0.342	2.68
Menstrual history	-1.065	1.014	1.105	0.293	0.35
Clinical Stage(1)	1.417	1.505	0.886	0.346	4.12
Clinical Stage(2)	1.259	1.364	0.851	0.356	3.52
Clinical Stage(3)	2.498	1.400	3.184	0.074	12.16
ER +	-1.267	1.368	0.857	0.355	0.28
PR+	0.991	1.383	0.514	0.473	2.69