Evaluation of HER2/neu oncoprotein in serum & tissue samples of women with breast cancer

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Background & objectives: The proto-oncogene HER2/neu has been extensively studied in breast cancer patients. Serum levels of HER2/neu by ELISA in breast cancer patients were compared with tissue HER2/neu expression and with other clinicopathological parameters with the aim to investigate whether the serum assay could replace the established tests (IHC/FISH) for HER-2 status.

Methods: Blood and Tru-cut biopsy samples were collected for determining HER2/neu status in 64 breast cancer patients. The tissue specimens were processed routinely and immunohistochemistry (IHC) for HER2/ER/PR (oestrogen/progesterone receptors) performed. Fluorescence *in-situ* hybridization (FISH) was performed on all HER2/neu 2 positive cases. Sixty age matched healthy females and females with benign breast disease were taken as controls for ELISA.

Results: Of the 64 breast cancer cases, 25 (39.1%) had elevated serum HER2/neu levels accompanied with increased tissue expression of HER2/neu receptors. On IHC, HER2/neu score was 3+ in 24 (37.5%) cases, 2+ in three (4.6%), 1+ in 18 (28.1%); while 19 cases (29.7%) showed no HER2/neu expression. Of the three 2+ cases on IHC, two showed amplification on FISH. Twenty one (32.8%) patients were ER positive and 17 (26.6%) were PR positive. There was a significant correlation (*P*<0.001) of serum HER2 concentration with tumour size, lymph node involvement, stage of disease and histological grade. Serum HER2/neu levels showed a negative correlation with ER status (*P*=0.047) but no correlation with PR status.

Interpretation & conclusions: The results suggest that elevated serum HER2 level was associated with a clinicopathological aggressive phenotype of breast carcinoma and was related to tissue HER2 overexpression. Therefore, serum HER2 may be useful for monitoring the course of the disease and response to treatment.

Breast cancer is the most common malignant tumour of women in urban India accounting for 27 per cent of all cancers in women[1]. According to Globocan 2012, 144,937 women were newly diagnosed with breast cancer in India in year 2012 and 70,218 women died of it.

An intensively studied proto-oncogene in human breast cancer is HER2/neu also known as c-erbB-2. It is a human epidermal growth factor receptor (member of epithelial HER1, HER3 and HER4 family). It is located on chromosome 17q21 and encodes a 185 kDa transmembrane glycoprotein receptor with tyrosine kinase cytoplasmic domain and a growth factor binding extracellular domain (ECD). The ECD of the receptor protein (also called as p105) can be cleaved from the cell surface by matrix metalloproteinases and then released into the blood[2]. Amplification and/or overexpression of the HER2/neu oncogene can be detected in 10-30 per cent of primary breast cancers[3,4]. The shed HER-2 ECD can be detected in the serum of approximately 15-40 per cent of breast cancer patients[2,5,6]. The ECD of the HER2 receptor which is shed into the blood of patients with breast cancer, can be measured in the serum by different techniques including enzyme linked immunosorbent assay (ELISA). Fluorescence *in situ* hybridization (FISH) and immunohistochemistry (IHC) identify amplification and over-expression of the HER2/neu gene and its product, the receptor, but each technique requires a high quality tissue sample, which may not always be available. On the other hand, estimation of ECD of the HER2 receptor shed in blood by ELISA is simple, easy to perform and cost-effective. Therefore, an association between serum and tissue HER2 levels was sought as serum HER2 estimations are advocated for follow up of breast cancer patients. The present study was undertaken with the aim to explore the clinical utility of serum HER2/neu estimation by ELISA in breast cancer patients and evaluate whether it could be used in place of FISH/IHC for diagnostic purpose.

Material & Methods

Study design: This was a hospital-based observational cross-sectional study conducted in the department of Pathology in collaboration with the department of Surgery, Lady Hardinge Medical College and Smt. Sucheta Kriplani Hospital, New Delhi, India, from November 2011 to March 2013. Study population included all women who presented in Surgery outpatient department with breast lump having a clinical suspicion of breast cancer. Sixty age matched healthy females and females with histopathologically or cytologically proven benign breast disease (BB) were taken as controls. Written informed consent was obtained from all cases and controls. The study protocol was approved by the institutional ethics committee.

Selection of cases: Sixty four consecutive cases of breast carcinoma diagnosed on fine needle aspiration cytology (FNAC) and subsequently confirmed on histopathological examination were included in the study. Only newly diagnosed cases of breast cancer including both primary breast cancer (PBC) and metastatic breast cancer (MBC) were included. Patients already on treatment were excluded. Clinicopathologic features such as age, menopausal status, tumour size, lymph node involvement, site of metastases, histological type, grade, oestrogen, progesterone and HER2/neu receptor status were noted. All patients were subjected to a complete diagnostic workup including imaging modalities like mammography, chest radiography, ultrasonography and computed tomography (CT) especially to detect metastases. Lymph node involvement and liver metastases were confirmed by FNAC. Blood samples (3 ml) were obtained from all the patients at the time of diagnosis before any treatment was started. Tru-cut biopsy was performed on all cases and the tissue specimens were fixed in 10 per cent buffered formalin and embedded in paraffin. Sixty age matched controls (for ELISA) including 30 healthy females, 20 women having fibroadenoma and 10 with fibrocystic disease were also included.

Serum HER2 assays: Blood sample (3 ml) was taken by venipuncture in plain vacutainer tubes under sterile conditions from both patients and controls. Sample was allowed to clot and was centrifuged at 1000g for 10 min. Serum was separated and stored in aliquots at -70°C. The sHER2 human ELISA kit (BioVendor-Laboratorní Medicína a.s., Czech Repubic) was used for the quantitative estimation of human soluble HER2 protein (soluble p97-115 HER2; the soluble circulating fragment of p185 HER2) in serum. The cut-off value used in this study was 30.5 ng/ml as per manufacturer's recommendation.

Immunohistochemistry (IHC): IHC staining of specimens was carried out on formalin fixed paraffin embedded breast cancer tissue using the HER2 antibody targeting a peptide on C-terminus of the 185 kDa HER2 protein (clone BV5, Diagnostic Biosystems, India), oestrogen receptor (ER) antibody (clone ID5, Diagnostic Biosystems, India) and progesterone

receptor (PR) antibody (clone 10A9, Diagnostic Biosystems, India) in 1:50 dilution each. College of American Pathologists (CAP) and the American Society of Clinical Oncology (ASCO) recommended guidelines for grading HER2/neu status was used[7]. Quick's ER/PR scoring system was used for grading oestrogen and progestrone receptors status[8].

FISH

All 2+ cases on IHC were subjected to FISH analysis using the PathVysion HER2 DNA Probe Kit (Abbott Laboratories, USA) according to the manufacturer's recommendations. The results were reported as the ratio between the average copy number of the HER2/neu gene and that of the chromosome 17 centromere, analysing 100 neoplastic nuclei. Specimens with a signal ratio of <2 with an average HER2 copy number < 4.0 signals/ cell were considered as non-amplified. A signal ratio of ≥ 2.0; with an average HER2 copy number ≥ 4.0 signals/ cell or even < 4.0 signals/ cell and specimens with a signal ratio of < 2.0; with an average HER2 copy number ≥ 6.0 signals/ cell were reported as amplified[7].

Statistical analysis: Normally distributed continuous variables were compared using the unpaired t test. Categorical variables were analysed using either the Chi square test or Fisher's exact test. One-way analysis of variance (ANOVA) and Pearson correlation was used for ascertaining significance of continuous variables among different groups.

Results

The patients' age ranged from 25 to 76 yr with a mean age of 46.1 ± 12.87 yr. Maximum number of cases (n=30, 46.9%) were between 36 to 50 yr of age. The clinicopathologic characteristics of the 64 preoperative breast cancer patients on the basis of serum HER2 ECD concentration are summarised in Table I. The age of women in control group ranged from 24 to 70 yr with a mean age of 42.4 ± 11.26 yr. Among the cases, 36 (56.2%) women were premenopausal. Among the controls, 35 women (58.3%) were premenopausal. Majority of cases (59/64, 92.18%) were invasive ductal carcinoma, NOS (not otherwise specified). The other five cases included one each of invasive lobular carcinoma, neuroendocrine carcinoma, metaplastic carcinoma (squamous cell carcinoma) and two cases of mucinous carcinoma.

Serum HER2/neu ECD: Mean serum HER2/neu levels in cases and controls were 38.22 ± 33.39 and $9.76 \pm$ 4.64 ng/ml, respectively. Although the mean level of HER2 ECD in controls with BBD (n=30) was slightly higher (14.64 ± 5.28 ng/ml) than in the healthy control (n=30) group (7.3 ± 3.1 ng/ml); it was well below the cut-off value of 30.5 ng/ml in all the controls. Twenty five (39.1%) breast cancer patients had elevated serum HER2/neu levels (33.2 -166.6 ng/ml). In breast cancer cases, the mean serum HER2 concentration in tissue HER2 positive cases (n=26) was 65.38 ± 37.92 ng/ml and in tissue HER2 negative cases (n=38) was 19.64 ± 7.34 ng/ml.

Significant correlation of serum HER2/neu concentration with tumour size was found, the levels increasing with the increase in size of tumour ($P<0.001$). The mean serum HER2/neu levels in patients having lymph node involvement (n=27, 59.24 ± 41.89 ng/ml) were significantly higher ($P<0.001$) than in those without lymph node involvement (n=37, 22.88 ± 10.50 ng/ml).

Four patients presented with metastatic disease at the time of diagnosis of breast cancer; two in liver and one each in ovary and lung. Patients with metastases (n=4) had markedly elevated serum HER2/neu levels (mean 102.44 ± 50.13 ng/ml) as compared to patients without metastases (n=60, 32.78 ± 25.53 ng/ml) and the difference was significant ($P<0.001$). There were two cases with local recurrence of the disease during the course of the study that showed increased serum levels of HER2 at the time of recurrence as compared to those at the time of initial diagnosis with levels rising from 92.0 and 73.8 ng/ml to 116.8 and 94.6 ng/ml, respectively. One patient developed skin metastasis later during the course of the study and showed a rise in serum HER2 concentration from 28.4 ng/ml at the time of initial diagnosis to 36 ng/ml at recurrence 15 months later.

There was a significant association between mean serum levels of HER2/neu and the clinical stage of disease with the levels rising with increasing stage ($P<0.001$). Moreover, the mean serum HER2/neu concentration was significantly raised in tumour of higher stage as compared to those of lower clinical stage (Table II). There was a significant association between histological grade and serum HER2 levels ($P<0.001$). All breast cancer patients with raised serum HER2 concentration had invasive ductal carcinomas (IDC). HER2 concentration was not found to be elevated in the other histologic types of breast carcinoma in the present study; and the number of samples in each type was very small.

Table I. Correlation between serum HER2/neu levels and clinicopathological features in breast cancer patients (n=64)				
Characteristics	No. of patients (%)	Serum HER2/neu level (ng/ml)		*P* value
		<30.5	>30.5	
Menopausal status				0.974
Premenopausal	36 (56.2)	22	14	
Postmenopausal	28 (43.8)	17	11	
Stage				0.003
I	4 (6.3)	4	0	
II	39 (60.9)	30	9	
III	16 (25)	4	12	
IV	5 (7.8)	1	4	
Tumour size, cm				0.053
<2	3 (4.7)	3	0	
2-5	39 (60.9)	27	12	
>5	22 (34.4)	9	13	
Lymph nodes status				<0.001
Negative	37 (57.8)	31	6	
Positive	27 (42.2)	8	19	
Tumour grade				0.010
Grade 1	11 (17.2)	10	1	
Grade 2	47 (73.4)	28	19	
Grade 3	6 (9.4)	1	5	
Histology				-
IDC, NOS	59 (92.8)	34	25	
others	5 (72)	5	0	
Metastasis				<0.01
Absent	60	39	21	
Present	4	0	4	
Oestrogen receptor				0.047
Negative	43 (67.2)	23	20	
Positive	21 (32.8)	16	5	
Progesterone receptor				0.202
Negative	47 (73.8)	28	19	
Positive	17 (26.6)	11	6	
Tissue HER2 receptor				<0.001
Negative	38 (59.4)	34	4	
Positive	26 (40.6)	5	21	
IDC, invasive ductal carcinoma; NOS, not otherwise specified				

Tissue HER2 status: Twenty four (37.5%) cases were HER2 positive on IHC with a score of 3+; three (4.6%) were equivocal with a score of 2+ and 18 (28.1%) cases had a score of 1+ while 19 (29.7%) showed no HER2/neu immunostaining. Two of the three cases with HER2 2+ score were amplified on FISH. Elevated serum HER2/neu levels were accompanied with increased tissue expression of HER2/neu receptors and the association was significant (*P*<0.001).

Hormone receptor status: Of the 64 patients, 21 (32.8%) were ER positive while 17 (26.6%) were PR positive. There was a significant negative correlation

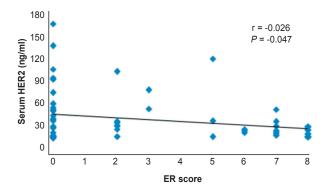| Table II. Serum HER2/neu levels in breast cancer stratified according to the clinical stage		
Stage	Serum Her2 (ng/ml)	
	Range	Mean ± SD
I + II	12.0 - 52.0	23.83 ± 10.67
III+IV	13.20 - 166.80	67.68 ± 43.78[*]
[*]P<0.001 compared to patients with stage I+II disease		

Table III. Comparison between HER2/neu overexpression by immunohistochemistry (IHC) and ELISA	
Status	No. of cases
IHC and ELISA positive	21
IHC negative ELISA positive	4
IHC and ELISA negative	34
IHC positive, ELISA negative	5
Total	64

(r=-0.026) between serum HER2/neu levels and ER status in breast cancer (*P*=0.047) (Figure). The HER2/neu levels were elevated in 60 per cent of cases where ER score was zero and not elevated on maximum ER score. There was no significant difference between the mean serum HER2 levels in PR positive and PR negative cases of breast cancer.

Comparison between HER2/neu in serum and tissue: Twenty one cases showed HER2 overexpression by both IHC and ELISA and 34 cases were found to be negative by both (Table III). Five cases showed overexpression of HER2/neu on IHC without increase in the serum levels while four cases were negative on IHC and positive by ELISA.

Discussion

This study showed a correlation between serum HER2 ECD concentration and tissue HER2 status as determined by IHC and FISH. Kandl *et al*[9] did not find any correlation between the serum and tissue HER2 levels probably because of the small sample size in their study. A couple of other studies also did not find a correlation between sHER2 ECD and tissue HER2 status[10,11], especially in primary breast cancer without distant metastases. However, a significant association have been reported especially in MBC disease[12,13].

Figure. Correlation of serum HER2 with oestrogen receptor (ER) score in breast cancer.

In the present study, high serum HER2 ECD levels were significantly associated with tumour size, clinical stage, nodal status, histological grade, distant metastasis, tissue HER2 status and negatively associated with oestrogen receptor status. Ludovini *et al*[14] found significant association between serum HER2/neu concentration and most of the clinicopathological parameters mentioned above except the tumour size having taken 2 cm as the cut-off size. The only discordant parameter in the study by James *et al*[15] was lymph node involvement. Imoto *et al*[16] did not find any significant association between raised serum HER2 and oestrogen receptor status. Tchou *et al*[13] reported a significant association only with tissue HER2 status and ER/PR negative status.

No significant relationship was found between other variables like age, menopausal status and progesterone receptor status like the other previous studies[10,17,18]. Pallud *et al*[19] reported a significant correlation between elevated serum HER2 and higher age. Kong *et al*[20] Ludovini *et al*[14], Lee *et al*[12] and others[12-14,20] observed significant association between serum HER2 levels and progesterone receptor status. Thriveni *et al*[21] found a positive correlation of serum HER2 with age, clinical stage and lymph node status and negative association with hormone status. There was no association with menopausal status. They also reported that serum HER2 levels were related to tumour size, histological grade, presence of comedo type carcinoma and tissue HER2/neu expression.

In the present study, patients with distant metastases (liver, lung and ovary) had markedly elevated serum HER2/neu levels as compared to those without metastases, similar to many previous studies[5,11-13,22,23]. In addition, serum HER2/neu levels were only moderately elevated with local recurrence but markedly with distant metastases as reported by Isola *et al*[22]. Anderson *et al*[24] found elevated serum HER2 levels significantly more often in patients with

distant metastasis than in patients with loco-regional recurrence (68 vs 19%). Thus, elevated serum HER2 levels are associated with HER-2 over-expression and amplification in breast cancer and reflect increased tumour aggressiveness and metastatic potential.

In the present study, four of the 64 cases were negative for HER2/neu overexpression on IHC but had raised serum HER2 levels while five were positive on IHC and negative on ELISA. Lack of tissue immunostaining may be related to the sensitivity of the method, tissue fixation playing an important role. The issue can be resolved to some extent by employing FISH analysis for ascertaining tissue HER2 status. However, it has been postulated that even a few HER2/neu positive cells within an essentially HER2 negative tumour can shed sufficiently detectable amount of HER2/neu protein in the serum[25].

Serial estimations of HER2 ECD, showing changes from the baseline level, may be useful for monitoring HER2/neu targeted therapy. Patients with decreased levels after receiving trastuzumab (with or without adjuvant chemotherapy) were more likely to have a higher response rate and longer disease free survival[25]. Similarly, Tchou *et al*[13] reported that in all patients who developed metastases, a steady rise in serum HER2 levels was noted before metastatic disease became clinically evident. Therefore, HER2 ECD has the potential of being a real-time biomarker for longitudinal assessments in breast cancer patients[25].

Thus, ELISA can be useful in assessing the response to therapy; estimating the tumour burden in the body; detecting occult metastases and selecting cases for trastuzumab therapy owing to its simple and inexpensive methodology. Moreover, serial estimations of HER2/neu by ELISA can be of value in the follow up of patients, often being the first clue to the detection of occult metastases. Immunohistochemistry, being a one-time procedure lacks this distinct advantage. It is recommended that ELISA be used in conjunction with IHC. IHC cannot be excluded, as it provides a permanent record of HER2/neu overexpression. Only ELISA may be performed when tissue is not available or is unsuitable for IHC and during follow up of the patient in whom tissue HER2 status has already been established.

The present study had some limitations. First, the number of patients included was small. Second, the patients were not stratified as PBC and MBC cases although the serum levels of HER2 were higher in patients having metastases at the time of diagnosis. It is known that serum HER2 testing is useful in monitoring treatment in MBC patients[11-13]. Third, serum HER2 levels were measured only once at the time of initial diagnosis before commencement of chemotherapy. The assay was repeated in a few cases at the time when they developed recurrence or distant metastasis. Longitudinal sequential measurements of HER2 ECD at definite time intervals are desirable. Fourth, relationship of serum HER2 levels with chemotherapy and/or trastuzumab therapy was not studied and the utility of serum HER2 assay as a prognostic tumour marker in follow up of patients was not assessed.

In conclusion, baseline levels of HER2 ECD may not correlate with tissue HER2 status. Serum HER2 assay cannot replace IHC/FISH testing but may complement the tissue assay in monitoring therapy of breast cancer patients by providing information that is lacking in IHC and FISH testing, such as early diagnosis of recurrence and metastases.

***Conflicts of Interest*:** None.

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