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Study the technical validity and feasibility of serum HER-2/Neu and its correlation with tissue HER-2 status in female breast cancer patients only

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ABSTRACT

Background: Breast cancer is one of the leading causes of death in women worldwide. The future burden of breast cancer is predicted to increase over 3 million new cases and 1 million deaths in 2040. In India, it is being increasingly diagnosed at a younger age as compared to the west. Human epidermal growth factor receptor 2 (HER-2) transmembrane protein, with a molecular weight 185 kDa, plays a key role in cell growth and its overexpression favors cell proliferation by inhibiting apoptosis. HER-2 extracellular domain, released into blood before appearing into tissue, can be easily detected by enzyme-linked immunosorbent assay (ELISA) using serum. HER-2 has many roles in assisting diagnosis, targeted therapy, and evaluating prognosis. **Aims and Objectives:** The study aimed to monitor the serum HER-2 level and its correlation with tissue HER-2 status in breast cancer patients. **Materials and Methods:** We performed a prospective study on technical validity and feasibility of serum HER-2 level by ELISA in breast cancer patients and its correlation with tissue HER-2 status by immunohistochemistry (IHC) method. **Results:** We found the serum ELISA level was raised and within the reference range in all breast cancer patients. A higher level of serum HER-2 level was seen in high tumor stage, high Nottingham grade, and with axillary lymph node metastasis. Serum HER-2 level was significantly higher in IHC score 3+ as compared to score 2+and 1+. **Conclusion:** This study concluded that serum HER-2 ELISA is a valid and feasible test. Association of serum HER-2 level with tumor stage; Nottingham grade; and axillary nodal metastasis was significant. The correlation between serum HER-2 and tissue HER-2 was found significant. Thus, serum HER-2 may be used as an early diagnostic and prognostic biomarker to guide in clinical practice.

Key words: Breast cancer; Serum human epidermal growth factor receptor 2/neuprotooncogene; Tissue human epidermal growth factor receptor 2

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INTRODUCTION

Breast cancer is the most diagnosed cancer in women and a leading cause of death worldwide. The future burden of breast cancer is predicted to increase to over 3 million new cases and 1 million deaths in 2040.[1] There are several methods to determine the presence of human epidermal growth factor receptor 2 (HER-2)/neuprotooncogene (Neu). Tissue HER-2 detection by immunohistochemistry (IHC) method has some limitations, such as loss of tissue antigenicity due to improper storage. In addition, every time, it is not possible to obtain tumor biopsies for follow-up and to monitor the course of disease.

HER-2 is a transmembrane protein with a molecular weight of 185 kDa. It plays a key role in regulation of cell growth, survival, and differentiation. The over expression of HER-2 favors cell proliferation by inhibiting cell apoptosis, which

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therefore leads to malignant tumor. The HER-2 protein contains three domains, namely, the extracellular domain (ECD), the intracellular segment with tyrosine kinase activity, and the transmembrane region. HER-2 ECD has been shown to be released into the blood from cancer cells and can be measured in serum. Serum HER-2 ECD serves as a highly sensitive and specific biomarker for breast cancer screening. More importantly, it has also been shown to be an independent prognostic factor. Different cutoff values have been proposed. Moreover, serum HER-2 ECD >62.5 pg/mL used as positive evaluation standard. In the past decade, serum HER-2 testing has not been used to guide in clinical practice. However, some studies suggested that serum HER-2 ECD might be promising biomarker in clinical practice for monitoring metastasis and recurrence.[2,3]

In this study, we evaluated the validity and feasibility of serum HER-2 level and its correlation with the tissue HER-2 status, especially predictive value of serum HER-2 in primary and metastatic breast cancer.

Aims and objectives

The main aim of this study was to monitor the values of serum HER2 in patients with primary breast cancer as well as in metastatic breast cancer. The study also aims to find correlation between serum HER 2 levels and expression of HER 2 in tumor cells.

MATERIALS AND METHODS

This prospective study was conducted in the Department of Pathology and Surgery OPD in M.L.B.M.C. Jhansi, for 14 months from July 2021 to September 2022 and 60 patients were included in the study after a thorough history and clinical examination and after getting institutional ethics committee clearance.

Inclusion criteria

- All female patients presented in the surgery outpatient department with breast lump having suspicion of breast cancer and confirmed by histopathological examination were included in the study.

Exclusion criteria

- All male patients with breast cancer were excluded from the study.
- Patients already on treatment were excluded from the study.

Written informed consent was taken from the willing patients. A brief history and examination were done. Blood sample (6 mL) in plain vacutainer for serum HER-2/Neu detection and biopsy tissue block for tissue HER-2 status was taken.[4]

Blood sample after centrifugation at 1000 g for 15 min and serum was separated. All the samples were evaluated in small batch by enzyme-linked immunosorbent assay (ELISA) (My Biosource) reader company Rayto model number RT2100C for quantitative estimation of soluble ECD of the HER-2/Neu protein in serum. For the quantification of human HER-2/Neu protein within the range of 62.5–4000 pg/mL was used. Serum HER-2 62.5 pg/mL was considered as baseline value.[5]

Preparation of all standards and reagents

Human HER-2 standard preparation

The lyophilized human HER-2 standard is reconstituted by adding 1 mL of standard/sample diluent to make the 10,000 pg/mL standard stock solution. Solution is allowed to sit at room temperature for 5 min after then gently vortex to mix completely.

Biotin-labeled detection antibody working solution preparation

The biotin-labeled detection antibody is diluted in 1:100 with the detection antibody diluent and mixed thoroughly.

Streptavidin-HRP working solution preparation

The streptavidin-HRP is diluted in 1:100 with the streptavidin-HRP diluent and mixed thoroughly.

Wash buffer working solution preparation

Entire contents (30 mL) of the wash buffer concentrate are poured into a clean 1000 mL graduated cylinder. Final volume to 600 mL is brought with glass-distilled or deionized water (1:20).

Procedure of ELISA

Step I

100 μL of each human HER-2 standard preparation and patient serum was added to well, covered and incubated for 90 min at room temperature. Then gently shaken. After discarding the solution plate was washed 3 times with wash buffer working solution.

Step II

100 μL of biotin-labeled detection antibody working solution is added into each well and plates were incubated at 37°C for 45 min. Then, plates were washed 3 times with wash buffer working solution.

Step III

Then, 100 μL of streptavidin-HRP working solution was added into each well and plate, incubated at 37°C for 45 min. Plates were washed with wash buffer working solution for 5 times. Then, 100 μL of TMB substrate solution was added to each well, incubated at 37°C in dark for 30 min.

Step IV

After adding 100 µL of stop solution in each well. Immediately color changes into yellow.

This is read at 450 nm in microplate reader within 30 min for O.D. absorbance.

Tissue HER-2 detection by IHC method

Quality control of IHC HER-2/Neu

A positive and negative control was run with every staining procedure performed for monitoring the correct performance of processed tissue and test reagents.

Immunohistochemical analysis

Biopsies of breast tumor tissue were fixed in 10% of neutral buffered formalin and embedded in paraffin blocks and automated IHC staining for HER-2/Neu was performed using anti-HER-2/Neu rabbit monoclonal antibody in Ventana benchmark machine. Each case was scored independently.

Scoring was done into four different categories according to the percentage of stained tumor cells.[6]

Score	HER-2/Neu overexpression assessment	Protein staining pattern (membranous staining)
0	Negative	No staining is observed, faint membrane staining <10% of tumor cells
1+	Negative	A faint or barely perceptible membrane staining is detected in >10% of tumor cells. The cells are only stained in part of the membrane
2+	Borderline	A weak to moderate complete membrane staining is observed in >10% of tumor cells or moderate to strong complete membrane staining in <10% of tumor cells
3+	Positive	A strong complete membrane staining is observed in >10% of the tumor cells

Statistical analysis was done by SPSS version 26.

RESULTS

In our study on 60 cases of female breast lump, blood samples were obtained from all cases for serum HER/neu detection and tissue HER-2 status was evaluated by immunohistochemical analysis (Figures 1-3).

The mean age of the patients was 51.9 years (±9.23). The age ranged from 43 years to 74 years. The patients mostly presented with a lump on the right side of the breast (56.66%), followed by left (35%) and bilateral (8.33%).

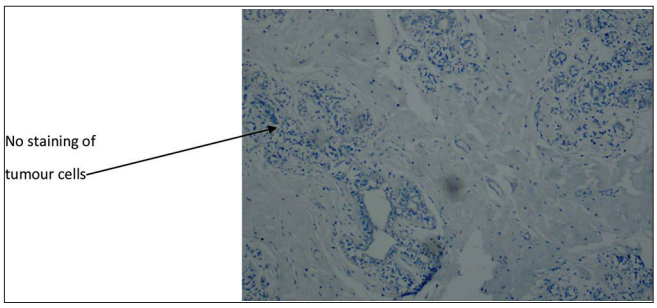

Figure 1: Invasive ductal carcinoma Grade-1 IHC Score 1+(×10) (Serum HER-2 level=259 pg/mL). IHC: Immunohistochemistry, HER-2: Human epidermal growth factor receptor 2

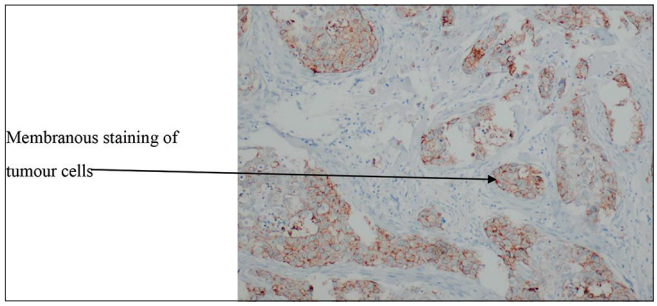

Figure 2: Invasive ductal carcinoma IHC HER-2 Score 2+(×10) (Serum HER-2 level=651 pg/mL). IHC: Immunohistochemistry, HER-2: Human epidermal growth factor receptor 2

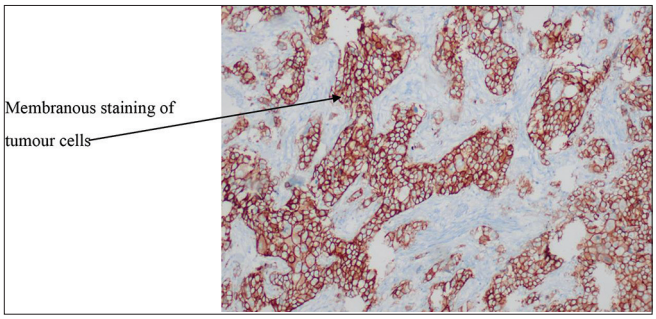

Figure 3: Invasive ductal carcinoma Grade-3 IHC HER-2 Score 3+ (×10) (Serum HER-2 level=1123 pg/mL). IHC: Immunohistochemistry, HER-2: Human epidermal growth factor receptor 2

Three-fourth of the patients had a single focality of the lump (n-47, 78.33%). Almost 63% of the patients had T2 size of lump, followed by 31.66% who had T1 size. The lump was in the upper outer quadrant of 61.66% (n=37) of the females. On palpation, the lump was firm in consistency and the border was ill-defined in all the women (n=60, 100%). All the patients of breast lump were diagnosed as invasive ductal carcinoma histologically (n=60, 100%) (Table 1).

The lumps were mostly of Grade 3 (Figure 4) (n=35, 58.33%), followed by Grade 1 (Figure 5) (n=16, 26.66%) and Grade 2 (Figure 6) (n=09, 15%). The base was not involved in any of the patients. The tissue HER-2/Neu was 3+ in 63% of the patients,

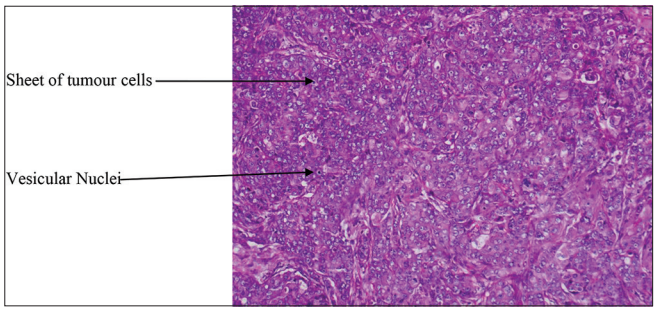

Figure 4: Invasive ductal carcinoma Grade-3 H and E (×10)

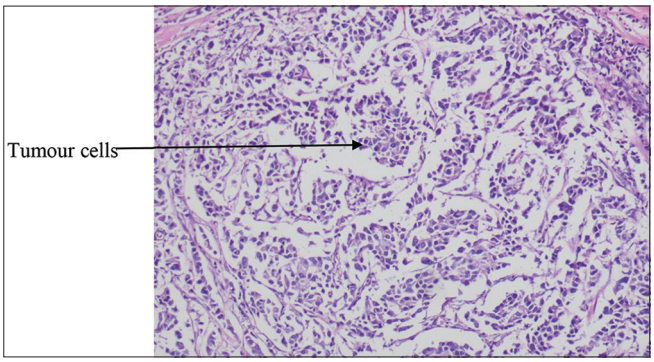

Figure 5: Invasive ductal carcinoma Grade-1 H and E (×10) invasive

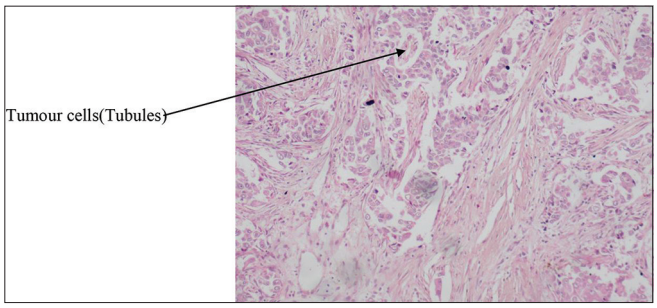

Figure 6: Invasive ductal carcinoma Grade-2 H and E (10×)

followed by 2+ in 32% of the patients. The axillary lymph nodes were involved in only 38%(n=23) of the patients (Table 2).

The mean serum HER-2/Neu level in cases of tissue HER-2/Neu positive cases is 1047.2 pg/mL whereas in cases of tissue HER-2 negative cases, the mean value of serum HER-2/Neu level is 430.2 pg/mL. On statistical analysis using unpaired Student's t-test, the difference was found to be statistically significant (P<0.001) (Table 3). Of the patients (n=36) with tissue HER-2 positive status, nine patients had elevated serum HER-2 level (≥1102.7 pg/mL) as defined by serum HER-2 values greater than the 75th percentile while out of 24 patients with tissue HER-2 negative status, six patients had raised serum HER-2 level (≥640.2 pg/mL) as defined by serum HER-2 values greater than the 75th percentile as shown in the box-plot (Figure 7).

Table 1: Clinical characteristics of the patients of breast cancer

Clinical parameters	No. of cases	Percentage
Site of breast lump		
Bilateral	5	8.33
Left	21	35.0
Right	34	56.66
Focality of lumps		
Unifocal	47	78.33
Multifocal	13	21.66
Size		
T1(≤2 cm)	19	31.66
T2(>2−<5)	38	63.33
T3(>5)	3	5.0
Quadrant involved		
Upper inner quadrant	14	23.33
Upper outer quadrant	37	61.66
Lower inner quadrant	2	3.33
Lower outer quadrant	7	11.66
Consistency of breast lump		
Firm with ill-defined border	60	100

Table 2: Histological parameters of the patients of breast cancer

Histological parameters	No. of cases	Percentage
Histologic type		
Invasive ductal carcinoma	60	100
Nottingham grade		
Grade 1	16	26.66
Grade 2	9	15.0
Grade 3	35	58.33
Lymphovascular invasion		
Present	02	3.33
Absent	58	96.66
Perineural invasion		
Present	00	00
Absent	60	100.0
Metastasis in axillary lymph node		
Absent	37	61.66
Present	23	38.33
Tissue HER-2 status		
Positive	36	60.0
Negative	24	40.0

HER-2: Human epidermal growth factor receptor 2, Neu: Neuprotooncogene

DISCUSSION

In breast cancer patients to reduce mortality and to increase survival of women, we should focus on early diagnosis and targeted therapy. Determining and monitoring the level of serum HER-2 are essential for early diagnosis and to start targeted therapy earliest and evaluating response to therapy.

In our study, analyzing the tumor size, tumors of <2 cm had serum HER-2 level in the range 260–300 pg/mL, size 2–5 cm had range 320–1000 pg/mL, and size of >5 cm had >1000 pg/mL. Similarly, in the study performed by James et al.[7], tumor size >5 cm (n=119) showed significantly (P>0.005) elevated levels of serum HER-2/Neu (19.29±47.4) compared to patients with smaller tumor size.

Table 3: Comparison of mean serum HER-2/Neu with tissue HER-2/Neu status

Tissue HER-2/Neu	No. of cases	Mean serum HER-2/Neu (pg/mL)(S.D.)	P-value
Tissue positive	36	1047.2 (100.8)	P<0.001(S)
Tissue negative	24	430.2 (179.8)	

HER-2: Human epidermal growth factor receptor 2, Neu: Neuprotooncogene

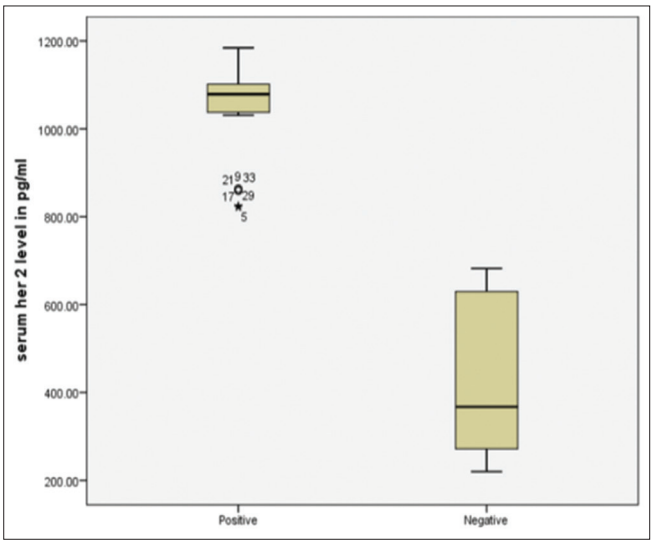

Figure 7: As shown by box-plot, the median serum HER-2/Neu level in tissue HER-2 positive patients is higher (1079 pg/mL) compared to tissue HER-2 negative patients (367.5 pg/mL). HER-2: Human epidermal growth factor receptor 2, Neu: Neuprotooncogene

In our study further while assessing the tumor grade in breast cancer patients, we found that Grade-3 patients (n=35/60) showed significantly raised serum HER-2 level in the range 800–1200 pg/mL followed by Grade 2 patients (n=9/60) showed serum HER-2 level 600–800 pg/mL and Grade-1 (n=16/60) showed serum HER-2 level 200–450 pg/mL.

The association of serum HER-2 level with grade was statistically significant (P<0.001). Similarly, a study performed by Shukla et al.[6] found a significant correlation of serum HER-2 level with histological grade.

Now, while studying the lymph node metastasis in breast cancer patients, 23 patients with axillary lymph node metastasis showed significantly higher serum HER-2 ECD concentration in range of (800–1200 pg/mL) as compared to 37 patients without nodal metastasis. Our finding suggested a significant correlation of serum HER-2 ECD with lymph node involvement also. These findings were consistent with the findings of study by Shukla et al.[6]

Furthermore, studying the correlation of serum HER-2 level with the tissue HER-2 status, out of 60 breast cancer cases, 36(60%) had elevated serum HER-2 levels accompanied with higher tissue HER-2 status. On IHC, HER-2/Neu score was 3+ in 36 cases, 2+ in 18 cases, and 1+ in six cases. In patients of 3+ score, serum HER-2 level was in the range of 1000–1200 pg/mL, followed by in 2+ score 300–700 pg/mL and least in 1+ score 200–250 pg/mL. We found significantly elevated serum HER-2 level in positive tissue HER-2 status as compared to negative tissue HER-2 status. There was a significant correlation (P<0.001) between serum HER-2 level and tissue HER-2 expression. Similarly, a study conducted on 94 breast cancer patients by Mokhtari et al.[7] in 2021, another study conducted on 64 breast cancer cases by Shukla et al.[6] in 2016 revealed a significant correlation of serum HER-2 level with tissue HER-2 expression.

In the previous studies, its clinical utility in breast cancer had limited values. Now, serum HER-2 may be used to assist in early diagnosis and to guide equivocal cases of breast cancer for close follow-up. It is not invasive as compared to biopsy for all follow-up cases after surgery, so patients compliance will be good.

ELISA being a simple and inexpensive methodology, it can be used in estimating tumor burden in the body, in assisting therapy response, detecting occult metastasis, and selecting cases for targeted anti-HER-2 therapy. Serial estimations of serum HER-2/Neu ECD can be done for patient follow-up. IHC does not have this distinct advantage. It is advised to use ELISA in conjunction with IHC. In the future, further studies are needed to serum HER-2 level to establish it as a promising early diagnostic as well as prognostic biomarker to guide in clinical practice and to assess targeted therapy response which would be helpful for clinicians. There is an intense need of research to monitor serum HER-2 level in equivocal cases of tissue HER-2 expression in suspected cases of breast cancer patients and give valuable advice for close follow-up.

In future, serum HER-2/Neu may be used as a promising biomarker in addition to a research marker to guide in clinical practice.

Limitations of the study

The study was of very short duration so the follow-up could not be done.

CONCLUSION

In this study, a significant correlation was observed between serum HER-2 level with tumor size, Nottingham

grade, and axillary lymph node metastasis. As the tumor size increases, metastasis occurs and histological grading (Nottingham grading) increases serum HER-2 level increases. Correlation of serum HER-2 with tissue HER-2 status was also statistically significant. We can assess serum HER-2 ECD level of many patients simultaneously by manual ELISA method which is a feasible test and gives valid results. This study concluded that serum HER-2 level can be used for early diagnostic and prognostic biomarker. The use of serum HER-2/Neu can improve the patient compliance. This study would help in early diagnosis and to start targeted therapy in younger patients of breast cancer which will reduce mortality and increase survival of young females. Serum-HER-2 detection is a cosmetically compliant test for young females. By analyzing serum HER-2 level, we can advise suspected and equivocal cases of breast cancer for close observation and follow-up. In biopsy-proven cases of breast cancer, we can predict prognosis by detecting serum HER-2 level by ELISA.

The present study had few limitations. Sample size was small and the study was of shorter duration. Hence, the follow-up of patients could not be done. This study should be done for longer duration so patients follow-up and compliance could be studied.

In the future, serum HER-2/Neu may be used as a promising biomarker in addition to a research marker to guide in clinical practice.

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Author's Contributions:
RS, SAK, and BS- Definition of intellectual content, literature survey, prepared first draft of manuscript, implementation of study protocol, data collection, data analysis, manuscript preparation and submission of article, concept, design, clinical protocol, manuscript preparation, editing and manuscript revision, design of study, statistical analysis and interpretation, literature survey, coordination, and manuscript revision.

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