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Original Article

Pathology Section

Reckoning the Status of HER2/neu Protein and Oncogene in Breast Cancer Specimens: Comparison of IHC Assay with FISH Assay at a Tertiary Care Centre, West India

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ABSTRACT

Introduction: Breast carcinoma is the most prevalent malignant neoplasm among women with more than one million new cases per year. The benefit of humanised anti-Human Epidermal growth factor Receptor 2 (HER2)/neu monoclonal antibody trastuzumab (Herceptin) in HER2/neu-positive breast cancers has been well documented. Immunohistochemistry (IHC) for protein overexpression and Fluorescence In Situ Hybridisation (FISH) for detecting HER2/neu gene amplification is widely used. Some laboratories use IHC as primary test with FISH for subset of cases while using FISH as primary investigation followed by IHC if needed is done by others.

Aim: To evaluate IHC assay in breast carcinoma cases for HER2/neu as screening test before FISH.

Materials and Methods: A cross-sectional descriptive type of observational study was conducted in the Department of Pathology and Multidisciplinary Research Unit, SMS Medical College and attached hospitals Jaipur, India, between April 2020 and December 2021. A total 122 samples of invasive breast carcinoma were included in study for IHC and FISH analysis. On paraffin embedded breast tumour tissue sections, IHC was performed using mouse monoclonal antibody targeting the intracellular domain of HER2/neu protein and FISH was implemented by dual colour probes targeting the HER2/neu gene on chromosome 17. Cases were classified according to HER2/neu status on IHC interpretation and FISH interpretation as per American Society of Clinical Oncology/the College of American Pathologists (ASCO/CAP) HER2/neu-2018 testing guidelines. Chi-square test, kappa coefficient and Z-test were applied for statistical analysis. The p-value <0.05 was considered significant.

Results: Of the 122 patients of Invasive Duct Carcinoma (IDC), the mean age was 50.3±12.7 years with a age range of 25-70 years. HER2/neu IHC score 3+ (overexpression) was seen in 19 (15.6%) cases while 2+ (equivocal) result was obtained in 84 (68.8%) cases and 19 (15.6%) cases showed negative (1+/0) HER2/neu expression. Out of 19 IHC positive cases, all cases were amplified by FISH and all 19 IHC negative cases, were non amplified by FISH. There were 84 IHC equivocal cases of which 37 (44%) cases were amplified, 45 (53.6%) non amplified and 2 (2.4%) cases were equivocal when analysed by FISH. Also, 11 (9%) cases were found Centromere Enumeration Probe (CEP) amplified in the study, they were negative or equivocal on IHC and none was IHC positive.

Conclusion: The study concluded that combined FISH and IHC methodologies could optimise information on HER2/neu status in breast cancer patients. Also, testing algorithm is emphasised where laboratories may use IHC as a screening method and FISH can be used as accurate and specific method in IHC (2+) equivocal cases. Thus, patients with HER2/neu status positive of IHC (3+) or FISH (gene amplified) can be proposed to be treated with herceptin (trastuzumab).

Keywords: Breast carcinoma, Fluorescence in situ hybridisation, Human epidermal growth factor receptor-2/neu, Immunohistochemistry

INTRODUCTION

Breast carcinoma is the most prevalent malignant neoplasm among women with more than one million new cases per year and its occurrence peaks ages between 40-60 years [1]. Tumour prognosis, aggressiveness and specific molecular pattern are determined by the molecular characterisation of the malignancy and for introducing new management for patient care. Erythroblastic oncogene B2 (ERB-B2) gene amplification through Fluorescence In Situ Hybridisation (FISH) is tremendously being confessed the most potentous and accurate assay for the treatment of breast cancer specimens [2]. The benefit of humanised anti-Human Epidermal growth factor Receptor 2 (HER2)/neu monoclonal antibody trastuzumab (herceptin) in HER2/neu-positive breast cancers has been well documented and noted for prolonged patient survival [3-5]. HER known as HER2/neu protooncogene is mapped on chromosome 17q12-21.32 region and encodes a 185 kDa transmembrane phosphoglycoprotein with tyrosine kinase activity [6]. The trastuzumab therapy treats patients with HER2/neu amplification by attaching itself to HER2/neu protein and blocking the ability of cancer cells to proliferate [7]. A 9-34% breast cancers cases, have been reported by researchers for the amplification [7].

Currently, for the quantification of HER2/neu, several techniques are available such as Reverse Transcription Polymerase Chain Reaction (RT-PCR), Southern blot analysis, Chromogenic In Situ Hybridisation (CISH) technique, IHC on paraffin-embedded tissues for HER2/neu protein detection and FISH technique [8]. IHC for protein overexpression and FISH for detecting gene amplification is widely used [9]. Some laboratories use IHC as primary test with FISH for subset of cases while using FISH as primary investigation followed by IHC if needed is done by others [10]. FISH is a robust and reliable complementary technique for the diagnosis of gene amplification, especially in resource-limited settings where extensive molecular assays may not be available to pinpoint the translocation partner. This technique is globally accepted for prognosis and to detect the response to targeted therapy. FISH amplification analysis is done as per American Society of Clinical Oncology/College of American

Pathologists clinical practice guideline (ASCO/CAP guidelines, 2018). HER2 signals per cell and HER2/Centromere Enumeration Probe (CEP)17 ratio are calculated and cases classified in five groups [11]. CEP17 value and its significance are not fully studied till date [12]. CEP amplification is said if CEP17 signals are more than three per cell while some studies take this cut-off as five per cell [10,12].

Study objectives:

- To investigate the HER2/neu IHC status in breast carcinoma samples.
- To investigate the HER2/neu gene amplification by FISH in breast carcinoma samples.
- Compare the results of HER2/neu status obtained by two modalities, in breast carcinoma samples.

As some laboratories use IHC as primary test with FISH for subset of cases, while, using FISH as primary investigation followed by IHC if needed, is done by others [10]. In resource limited settings of the present study laboratories performing expensive test like FISH in all the cases gives extra financial burden in patient care. The test is mandatory to be done to ensure correct targeted therapy. So, study was carried out to see if IHC could be used in all the cases followed by FISH in subset of IHC equivocal cases only, in the present study settings, without affecting the individual patient care.

MATERIALS AND METHODS

The cross-sectional descriptive type of observational study was conducted in the Department of Pathology and Multi-Disciplinary Research Unit, SMS Medical College and attached hospitals Jaipur, India, between April 2020 and December 2021, on 122 samples of breast carcinoma. Breast carcinoma cases underwent surgery at the Department of Surgical Oncology and samples were referred for histopathology fixed in 10% Neutral Buffered Formalin (NBF) to the Department of Pathology. IHC and FISH were done at Department of Pathology and multidisciplinary research unit, respectively, after obtaining the informed written consent for FISH analysis. The study approval was granted by Institutional Ethical Committee (IEC No. 316/MC/EC/2020) and study was performed in a manner to conform with the Helsinki declaration of 1975, as revised in 2000 and 2008. Samples were fixed strictly for 6-72 hours in 10% NBF and processed for histopathology. Analysis for IHC and FISH was done till April 2022 and then data was analysed.

Inclusion criteria: Properly labelled biopsies, lumpectomy samples and Modified Radical Mastectomy (MRM) samples diagnosed on histopathology as Invasive Duct Carcinoma of Breast-No Special Type (IDC-NST) were included in the study.

Exclusion criteria: Improperly fixed tissue, core biopsies with inadequate tumour cells and histological type other than IDC-NST were excluded from the study.

Sample size calculation: Sample size was calculated at 80% study power and alpha error of 0.05 assuming 82% concordance of HER2/neu status between IHC and FISH tests in breast cancer specimens as found in the study by Singhai R et al., [9]. At absolute allowable error of 7%, 120 breast cancer samples were required as a sample size.

Study Procedure

Immunohistochemical (IHC) analysis: The IHC to study HER2 protein overexpression was performed on Formalin Fixed Paraffin Embedded (FFPE) tissues. A 2-3.5 µm thick tissue sections on poly-L-Lysine coated slides were taken. After deparaffinisation, antigen retrieval and blocking of endogenous peroxidase by Horseradish Peroxidase (HRP), HER2/neu immunostaining was performed using rabbit monoclonal anti-human c-erbB-2 oncoprotein as primary antibody {Prostaglandin E2 receptor 3 (EP3) clone, Biocare} at 1:80 dilution, Oestrogen Receptor (ER) (SP1 clone, Biocare) and Progesterone Receptor (PR) (SP2 clone, Biocare). Primary antibody binding was checked by quick-staining, Labelled Streptavidin-Biotin System (LSAB, Biocare) which is further followed by the addition of chromogen called diaminobenzidine (DAB, Pathnsitu). Already known positive cases were run as positive controls in each batch for ER, PR and HER2/neu. Allred scoring system was used to quantify the expression of ER and PR [13]. HER2 assessed as per ASCO/CAP guidelines and scored for HER2/neu. Score of 1+ were reported as negative, 2+ as equivocal and 3+ as positive and were further processed for FISH analysis [9].

Fluorescence In Situ Hybridisation (FISH): Scrutiny of FISH was performed by using the Zytolight, SPEC ERBB2/CEN 17 dual colour probe (PL8) (ZytoVision, GmbH, Germany) for the qualitative identification of human ERBB2 gene amplification. There were two fluorescent-labelled dual colour probes: HER2/neu specific Fluorescein Isothiocyanate (FITC)-labelled green (locus specific identifier) for the HER2 gene locus 17q12-q21 and Chromosome Enumeration Probe (CEP) 17p11-q11 specific for the alpha satellite centromeric region D17Z1 of chromosome17 rhodamine-labelled, orange probe (ZytoVision, GmbH, Germany). A 2-3.5 µm thick FFPE sections were mounted on poly-L-Lysine coated slides, and allowed to dry. The slides after overnight incubation at 56ºC were deparaffinised in xylene and rehydrated. Washed the slides, treated with protease and applied probe after dehydration. Posthybridisation wash was given and dried slides completely in dark and 10 µL 4′,6-Di Amidino-2-Phenyl Indole (DAPI), was applied. Slides were evaluated for HER2/neu gene amplification.

Two blind folded observers for each other's findings, IHC results and clinical details scanned the slides (Axio Imager Z2, Zeiss microscope; filters FITC, DAPI, Spironaphthoxazine (SPO), aqua and dual; Plan Apochromatic objective) at 10X for non overlapping invasive nuclei showing defined nuclear borders on DAPI filter and areas were chosen to count CEP17 and HER2/neu signals at 63X oil magnification. H&E stained slides were always assessed beforehand to ensure no loss of representative tissue. Uniform staining and normal cell signals were used as internal control of staining. At least 20 invasive carcinoma (excluding DCIS component) nuclei showing no overlap were assessed by each observer and the number was increased to atleast 60 nuclei in case of intratumoural or intertumoural heterogeneity. There were two cases (1.6%) where there was discordance between two observers and opinion from third observer was sought. FITC (HER2) and SPO (CEP-17) signals were counted in their respective filters for each nucleus continuously using fine focus to achieve as accurate counts as possible. Nuclei with single colour signals, <2 CEP signals were not included in assessment. Clusters with more than 10 HER2 signals were counted as 10 for calculations [10]. Signals with diameter distance less than a signal diameter were counted as one. Total HER2/neu and CEP-17 signals, mean HER2 and CEP signals per nuclei and HER2 to CEP ratio were analysed [11,12]. Clinicopathological parameters like age, gender, histological grade, tumour staging, lymph node status, ER-PR-HER2/neu status on IHC were evaluated and comparison of IHC and FISH results for HER2/neu was done.

STATISTICAL ANALYSIS

Statistical analysis was done using Chi-square test, kappa coefficient and Z-test. The p-value <0.05 was considered statistically significant. Kappa coefficient and Chi-square tests to show the association and data analysed using Statistical Package for Social Sciences (SPSS) software version 20.0. Accuracy of HER2/neu expression by IHC was calculated assuming FISH as gold standard. Z-test was used to determine significant difference of inconclusive diagnoses by two test modalities.

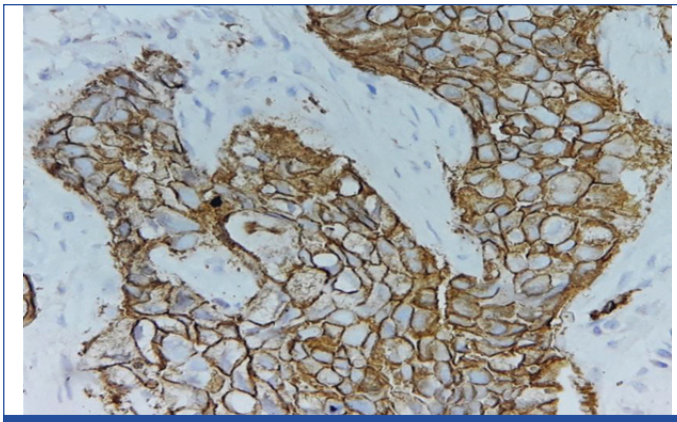
RESULTS

Of the 122 patients of IDC, mean age was 50.3±12.7 years (range 25-70 years) [Table/Fig-1]. A 70 (57.3%) patients were of age less

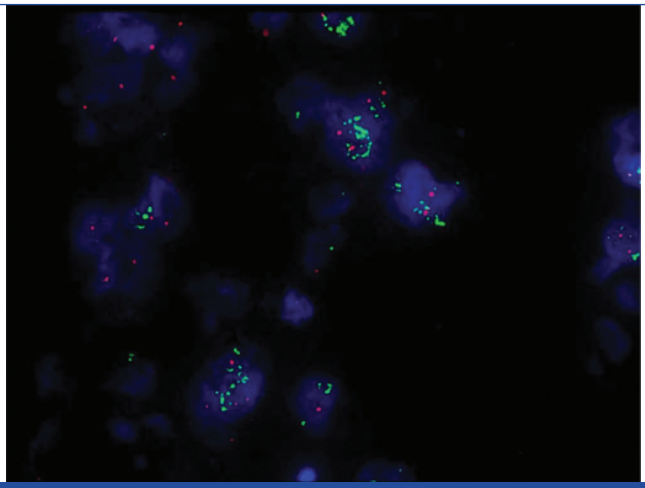
than or equal to 50 years and 52 (42.7%) were above 50 years of age. None of the two age groups was prone to amplification of HER2/neu. Out of these 118 (96.7%) were females and 4 (3.3%) were male patients. All the four male patients included in study had no amplification of HER2/neu. Samples included were properly labelled biopsies 34 (27.86%), lumpectomy 01 (0.82%) and MRM 87 (71.31%), which were diagnosed on histopathology as IDC-NST. An 87 (71.31%) MRM was the most common sample type received and was evaluated for both tumour pathological staging and lymph node status. One (0.82%) lumpectomy specimen was evaluated for tumour size but not lymph node status. A total of 34 (27.86%) biopsies received were not evaluated for tumour staging and lymph node status. Maximum 101/122 (82.7%) cases were of histological grade II and maximum cases 66/88 (75%) MRM and lumpectomy specimens were of T2 pathological tumour staging. A 44/87 (50.6%) MRM cases had no lymph node metastasis at the time of evaluation. Predominant cases 78/122 (63.4%) and 91/122 (74.6%) were negative for ER and PR, respectively. A 19/122 (15.57%) IHC positive cases for HER2/neu were all amplified on FISH [Table/Fig-2,3], 19/122 (15.57%) IHC negative cases were all gene non amplified [Table/Fig-4,5] and out of 84/122 (68.8%) IHC equivocal cases 37/84 (44%)

Clinicopathological parameter	Subgroup	FISH HER2/neu expression	
		Amplified n (%)	Non amplified n (%)
Age (in years)	≤50 years	36/122 (29.5)	34/122 (27.8)
	>50 Years	21/122 (17.3)	31/122 (25.4)
Sex	Male	0/122 (0)	4/122 (3.2)
	Female	57/122 (46.8)	61/122 (50)
Nature of tissue	Biopsy	21/122 (17.3)	13/122 (10.6)
	Lumpectomy	0/122 (0)	1/122 (0.8)
	MRM	36/122 (29.5)	51/122 (41.8)
Histological grading (Scarff Bloom Richardson's scoring)	Grade I (3-5)	4/122 (3.3)	4/122 (3.3)
	Grade II (6-7)	48/122 (39.3)	53/122 (43.4)
	Grade III (8-9)	5/122 (4.1)	8/122 (6.6)
T-staging	T1	2/88 (2.2)	7/88 (8.0)
	T1c	0/88 (0)	2/88 (2.2)
	T2	30/88 (34.1)	36/88 (41.0)
	T3	3/88 (3.4)	3/88 (3.4)
	T4	1/88 (1.1)	4/88 (4.6)
N-staging	N0	19/87 (21.8)	25/87 (28.8)
	N1	5/87 (5.7)	9/87 (10.4)
	N1a	3/87 (3.4)	3/87 (3.4)
	N2	7/87 (8.0)	5/87 (5.7)
	N2a	1/87 (1.2)	2/87 (2.3)
	N3	2/87 (2.4)	5/87 (5.7)
	N6	0/87 (0)	1/87 (1.1)
ER status on IHC	None (0-1)	34/122 (27.9)	32/122 (26.2)
	Weak (2-3)	7/122 (5.8)	5/122 (4.1)
	Intermediate (4-6)	9/122 (7.3)	9/122 (7.3)
	Strong (7-8)	7/122 (5.8)	19/122 (15.6)
PR status on IHC	None (0-1)	40/122 (32.8)	35/122 (28.7)
	Weak (2-3)	8/122 (6.6)	8/122 (6.6)
	Intermediate (4-6)	2/122 (1.6)	8/122 (6.6)
	Strong (7-8)	7/122 (5.8)	14/122 (11.4)
HER2/neu on IHC	Negative (0/1+)	0/122 (0)	19/122 (15.6)
	Equivocal (2+)	38/122 (31.1)	46/122 (37.7)
	Positive (3+)	19/122 (15.6)	0/122 (0)

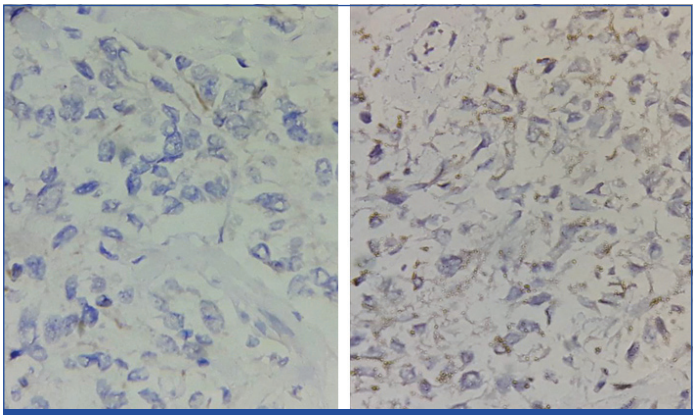
[Table/Fig-1]: Association of various clinicopathological parameters with HER2 neu expression.



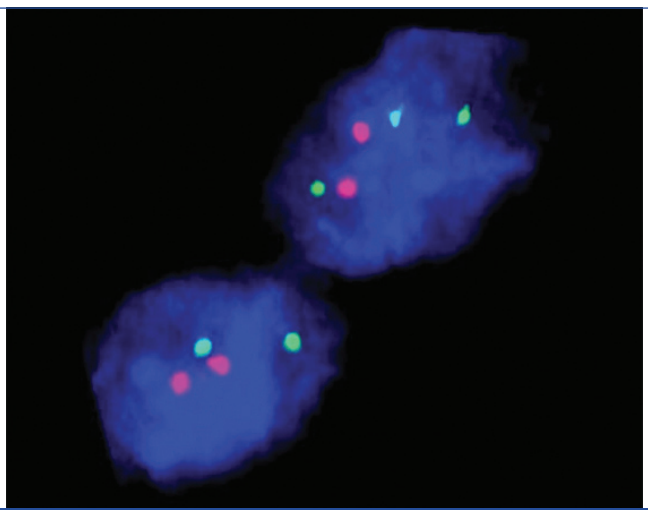
[Table/Fig-2]: HER2/neu positive (3+) (IHC, 40X).



[Table/Fig-3]: Nuclei showing HER2/neu gene amplification (HER2 FITC labelled green and CEP17 rhodamine labelled red). (FISH Dual Filter).



[Table/Fig-4]: HER2/neu negative (0/1+) (IHC, 40X).



[Table/Fig-5]: Lack of amplification of HER2/neu gene (HER2 FITC labelled green and CEP17 rhodamine labelled red) (FISH Dual filter).

were HER2/neu gene amplified and 45/84 (53.6%) were gene non amplified, while, 2/84 (2.4%) remained equivocal on FISH for gene amplificaton and were evaluated further.

Of the 84/122 (68.8%) patients equivocal for HER2\neu on IHC, 37/84 (44%) were HER2 gene amplified, 45/84 (53.6%) were non amplified and 2/84 (2.4%) had equivocal results by FISH [Table/Fig-6]. So, FISH provided 97.62% extra diagnostic yield.

HER2 protein expression by IHC		FISH			
		Amplified	Non amplified	Equivocal	Total
Conclusive cases on IHC	Positive	19	0	0	19
	Negative	0	19	0	19
Non conclusive cases on IHC	Equivocal	37	45	2	84
Total		56	64	2	122

[Table/Fig-6]: Comparison of FISH results with IHC.
Values are presented as n

Inconclusive or equivocal cases on IHC and FISH were statistically significant with 84/122 (68.8%) cases equivocal on IHC, while, only 2/122 (1.6%) cases equivocal on FISH with p-value <0.05. Poor agreement was observed between both the modalities (kappa coefficient=0.087, p-value <0.001) [Table/Fig-7]. Thus, FISH had provided 100% diagnostic yield as compared to IHC (45.90%).

Technique	Inconclusive diagnosis	Proportion	Z-test	95% CI of difference	p-value
IHC	84/122	0.689	10.868	0.5531 to 0.7929	**<0.001**
FISH	2/122	0.016			

[Table/Fig-7]: Inconclusive diagnosis by FISH and IHC.
p-value in bold font indicates statistically significant values

These 2 (2.4%) cases were re-evaluated as per ASCO/CAP guidelines and were concluded one as amplified and another non amplified. Of the two FISH equivocal cases one was CEP amplified with CEP/Cell value 3.35 and HER2/Cell 4.78 with HER2/CEP ratio 1.43. Breast Imaging Reporting and Data System (BIRADS) score was 5 and microscopically was grade III with RB score 8. Three lymph nodes were positive for tumour metastasis and were negative for both ER and PR on IHC. Thus, the case has an average HER2/cell of ≥4.0 and <6.0 and the HER2/CEP17 ratio was <2.0 and was taken for additional workup. IHC results were 2+ and recounting by FISH was done on 60 invasive tumour cells by an additional observer blinded to previous results. The count remained same as of ≥4.0 and <6.0 and the HER2/CEP17 ratio is <2.0, the diagnosis given was HER2 negative with the comment as per guidelines that it is uncertain whether the patient will get benefit from HER2 targeted therapy in the absence of protein overexpression.

The second FISH equivocal case was CEP amplified with CEP/Cell value 4.8 and HER2/Cell 8.8 with HER2/CEP ratio 1.8, was histologically grade II with RB score 6. No lymph nodes metastasis was noted and was negative for both ER and PR on IHC. Thus, the case has an average HER2/cell of >6.0 and the HER2/CEP17 ratio was <2.0 and was taken for additional workup. IHC results were 2+ and recounting by FISH was done on 60 invasive tumour cells including an additional observer blinded to previous results. The count remained same as of average HER2/cell of >6.0 and the HER2/CEP17 ratio <2, so the final diagnosis given was HER2 positive compliant with ASCO/CAP 2018 guidelines.

An 11/122 (9%) cases were CEP amplified with CEP17/Cell ≥3 and 111/122 (91%) were CEP non amplified. CEP amplified vs non amplified group had average HER2/neu per cell 7.97±1.64 and 4.85±3.03, with 11/11 (100%) cases positive for HER2/neu (≥4 HER2/neu per cell) while only 47/111 (42.3%) HER2/neu amplified, respectively and this value was statistically very significant (Unpaired t-test had two tailed p-value <0.001). CEP amplified group had no IHC3+ case, while non amplified group had 19/111 (17%) cases with HER2/neu score 3+. Histological grade III was found in 2/11(18%) CEP amplified cases, while 11/111 (9.9%) CEP non amplified cases. A 3/11 (27%) CEP amplified cases had lymph node metastasis while 63/111 (57%) CEP non amplified cases had lymph nodes positive for metastasis, however these values presented some inclination but were not statistically significant.

DISCUSSION

Healthy breast cells show low protein expression and contain just two copies of the HER2 gene per cell located on long arm of chromosome 17(17q12-21.32) and encodes pl85 oncoprotein which is a receptor tyrosine kinase that can be associated with multiple signal transduction pathways [9,14]. Current management of patients of carcinoma of the breast depends on the pathology and status of the prognostic markers. Adjuvant therapy including HER2 antagonist is strongly associated with improved survival [15]. Thus, evaluation of HER2/neu status using fine technical skills, robust techniques, precise interpretation has become pivotal in determining patient's eligibility for trastuzumab treatment and to avoid unnecessary cardiotoxic side effects if not showing true amplification or overexpression [9,10,14].

Clinical laboratories assess HER2 status in formalin fixed and paraffin embedded specimen using either IHC or Fish as primary test [10,14]. FISH is considered as a gold standard because of its sensitivity and specificity but has disadvantages of requirement of expensive fluorescence microscope equipped with multi bandpass florescence filters and slides fades so fast that keeping a permanent record is not practical [16]. Compared with FISH, IHC is widely used in developing countries and it is cheaper and morphology is clear and keeping records is easier [9].

Out of these 84 patients 37 were amplified, 45 were non amplified and two were found equivocal for HER2 neu on FISH. So, FISH provided 97.62% extra diagnostic yield. The two patients found equivocal on FISH were converted one to negative and another to positive on reflex testing. Thus, FISH had provided 100% diagnostic yield as compared to IHC (45.90%) [Table/Fig-7].

Some laboratories use FISH as primary test while others use IHC as primary and FISH as supplementary test. Present study found results in favour of using IHC as screening test and FISH as complimentary test in IHC equivocal cases with the available resource limited laboratory settings.

Studies by Goud KI et al., Panjwani P et al., and Murthy SS et al., show higher rate of concordance between IHC and FISH interpretation in cases which was either positive or negative on IHC testing whereas shows discordance in IHC equivocal cases same as seen in present study [14,17,18].

CEP17 copy number is used in the interpretation of HER2 status. A mean of CEP 17 ≥3 is adopted as a threshold as for amplification of chromosome 17. The study observed 11/122 (9%) CEP versus 111/122 (91%) CEP non amplified cases with groups presenting average HER2/cell 7.97±1.64 and 4.85±3.03, respectively. All the cases in CEP amplified group where HER2 amplified ≥4 HER2\neu per cell. CEP amplified group had no IHC HER2 3+cases. While non amplified group had 17% HER2/neu 3+ score cases. On lymph nodes status assessment, 3/11 (27%) CEP amplified cases had lymph node metastasis while 63/111 (57%) CEP non amplified cases had lymph nodes positive for metastasis. While study by Davies V and Voutsadakis IA revealed 56.4% lymph node positive cases in CEP amplified group while 38.8% lymph node positive cases were seen in CEP non amplified group [12].

Limitation(s)

Other than main findings of the study, comment on additional findings of CEP amplification in the present study comes with limitations of small sample size. Besides its effect on HER2/neu per cell, conclusion was not possible, while comparing two groups

for other clinicopathological parameters. Confirmation on clinical significance of CEP amplification needs further evaluation with larger sample size.

CONCLUSION(S)

In order to improve the therapeutic effect of Herceptin in HER2/neu positive breast cancers, we propose HER2/neu testing algorithm for resource limited laboratories in developing countries like India where penetration of FISH testing is still less in society. Our study reiterates the use of IHC as screening modality due to cost effectivity, easy availability and as it has good concordance with FISH in IHC conclusive cases. Present study found that re-confirmation of the IHC results by FISH is not required in IHC conclusive cases. Though FISH is very accurate and highly specific method due its availability and cost compared to other molecular diagnostic tests we can use this test accurately as supplementary test in IHC equivocal (2+) cases. Thus, use of IHC as screening modality before FISH, is found acceptable in the laboratory settings of limited resources. CEP17 amplification needs further exploration and its clinical effect on patients must be assessed to understand the therapeutic effects in future.

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