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Evaluation of HER-2/*neu* status in breast cancer specimens using immunohistochemistry (IHC) & fluorescence *in-situ* hybridization (FISH) assay

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***Background & objectives*: Fluorescence *in situ* hybridization (FISH) is increasingly being recognized as the most accurate and predictive test for HER2/*neu* gene amplification and response to therapy in breast cancer. In the present study we investigated HER-2/*neu* gene amplification by FISH in breast carcinoma tissue specimens and compared the results with that of immunohistochemical (IHC) analysis.**

***Methods*: A total of 90 breast carcinoma tissue samples were used for immunohistochemical (IHC) and FISH analysis. IHC was performed by using mouse monoclonal antibody to the intracellular domain of HER-2/*neu* protein. Each slide was scored in a blinded fashion by two pathologists according to the manufacturer's recommended criteria. FISH analysis was performed on paraffin embedded breast tumour tissue sections. The polysomy for centromere 17 (Spec green signal) was read as green signals less than 4 as moderate polysomy, and more than 4 as highly polysomy.**

***Results*: Thirty of the 90 patients had negative results by IHC and FISH. Of the 28 patients with the score of 2+ by IHC, 20 were FISH positive for HER-2/*neu* gene amplification, three were FISH negative and five patients showed equivocal (1.8-2.2) results by FISH. These five cases were retested for IHC and FISH on different paraffin embedded tissue blocks, and all five were found positive for HER-2/*neu* gene amplification. Twenty five patients with the score of 3+ by IHC were FISH positive for HER-2/*neu* gene amplification (>2.2). Seven cases with the score of 3+ by IHC were FISH negative for HER-2/*neu* gene amplification (>2.2), and showed polysomy of chromosome number 17 high polysomy > 4.**

***Interpretation & conclusions*: Our results indicated that HER-2/*neu* status by FISH should be performed in all cases of breast tumour with a 2+ score by IHC. Cases demonstrating a 3+ score by IHC may be subjected to FISH to rule out polysomy of chromosome 17 which could be falsely interpreted as HER-2/*neu* overexpression by IHC analysis. There is also a need for establishing a clinically validated cut-off value for HER-2/*neu* FISH amplification against IHC which may be further compared and calibrated.**

Key words Breast cancer - HER2/*neu* gene amplification - fluorescence *in situ* hybridization (FISH) - immunohistochemistry (IHC)

Breast cancer ranks as the second most common cancer for women in India and its incidence peaks between the ages of 40 and 50 yr, with a mean age of occurrence at 47 yr[1,2]. Since the first report of *erb*-b2 as a poor prognostic factor for breast cancer in 1987 [3], the significance of HER-2/*neu* oncogene as an adverse prognostic factor has been noted in many other cancers, such as ovary, lung, stomach, and pancreas[3-6]. Amplification of the *erb*-b2 gene or over-expression of the *erb*-b2 protein has been detected in 10 to 30 per cent of breast cancers[3-13] and, together with the associated topoisomerase IIα (*TOP2*) gene aberrations, shows no change over time[4-6] after treatment, and no difference between primary and metastatic lesions[5,6].

Several techniques like immunohistochemistry (IHC) on paraffin-embedded tissues for HER-2/*neu* protein detection, Southern blot analysis, a reverse transcription-polymerase chain reaction (RT-PCR) technique chromogenic *in situ* hybridization (CISH) technique and fluorescence *in situ* hybridization (FISH) to quantify HER2/*neu* are now available. HER-2/*neu* testing is used as a prognostic marker to determine the aggressiveness of breast cancer tumour. Serum HER-2/*neu* level is sometimes evaluated to establish a baseline and if elevated, is used to monitor cancer treatment. However, this method is not widely used because HER-2/*neu* levels are elevated only when a large tumour is present at an advanced stage; so early cancers are likely to be negative for serum HER-2/*neu*. FISH is widely used for diagnosis as well as to monitor the response to therapy, such as hormone therapy and chemotherapy. In this study we investigated HER-2/*neu* gene amplification by FISH in breast carcinoma specimens and compared the results of FISH analysis with IHC.

Material & Methods

In this retrospective study, paraffin-embedded tissue sections of breast carcinoma from 90 consecutive female patients who had undergone surgery at the Department of Oncology between July 2008 - April 2009 and referred to the Department of Molecular Biology and Cytogenetics, Apollo Health City, Hyderabad, India, for FISH analysis were included in the study. The mean age of patients ranged between 30-50 yr (47.94 ± 13.7). The histopathological diagnosis of breast carcinoma was established by standard light-microscopic evaluation of sections stained with Hematoxylin and Eosin in each case and diagnosed as invasive ductal carcinoma grade I, II and III. Estrogen and progesterone receptor (ER, PR) status was also noted.

Immunohistochemical (IHC) analysis: IHC of HER-2/*neu* protein was performed on 3 to 4 µm thick paraffin embedded tissue sections placed on poly-L-Lysine coated slides. After deparaffinization and blocking of endogenous peroxidase, HER-2/*neu* immunostaining was performed using rabbit anti-human c-erbB-2 oncoprotein as primary antibody (Dako, Copenhagen, Denmark) at 1 : 100 dilution. Binding of the primary antibody was checked by Dako Quick-Staining, Labelled Streptavidin–Biotin System (LSAB; Dako, USA), followed by the addition of diaminobenzidine (DAB) as a chromogen. Each slide was scored in a blinded fashion by two pathologists according to the manufacturer's recommended criteria. The immunostaining was read in a semiquantitative manner and graded as follows: 0, 1+, 2+ and 3+. Intensity scores of 0 or 1+ were designated as negative expression and 3+ were designated as positive expression for HER-2/*neu*. Scores of 2+ were taken as equivocal cases, which were further recommended for FISH analysis.

Fluorescence in situ hybridization (FISH) analysis: FISH analysis was performed using the PathVysion HER-2 probe kit (Vysis, USA). There were two fluorescent-labelled probes:LSI (locus-specific identifier) HER-2 specific for the HER-2 gene locus (17q11) and CEP (chromosome enumeration probe) 17 specific for the α satellite DNA sequence at the centromeric region of chromosome 17. Paraffin sections of 3-4 mm thickness using a microtome were cut and were floated in a protein- free water bath at 40^{0}C. The sections were mounted on poly-L-Lysine coated slides and allowed to dry. The slides were kept in oven overnight at 56^{0}C. The slides were deparaffinized in xylene at room temperature for 20 min and dehydrated in 100 per cent ethanol for 15 min at room temperature and air dried. The slides were treated with pretreatment solution (sodium thiocyanite) and protease solution for 15 min, and were dehydrated with 70, 80 and 100 per cent alcohol for 5 min each and air dried. The probe was denatured at 80^{0}C for 5 min and applied on to the slide and cover slipped, and placed in humidified chamber for overnight incubation. After that post-hybridization washes were given with 0.4 per cent 2XSCC (sodium saline citrate) 40 at 37^{o}C. After removing cover slips the slides were dipped in post- hybridization buffer for 18 sec, dried completely in dark and 10 µl DAPI was applied and coverslip is gently placed. The slides were screened under florescent microscope (Olympus, USA) using appropriate filters (DAPI, FITC, TRITC dual and triple band pass filters). Signals were counted in at least 200 cells for both the HER-2/*neu* gene and chromosome

17 centromere signals under oil immersion at x 1000 magnification using recommended filters. Results were expressed as the ratio of HER-2/*neu* signal (orange) to centromere 17 signal (green) and the readings were read as follows: the expected ratio 1-1.8 indicates no gene amplification (negative), a ratio of >2.2 as HER-2/*neu* gene amplification (positive), and a ratio between 1.8-2.2 as equivocal cases. The polysomy 17 was also recorded in the cells as four spec green signals as moderate polysomy and >4 spec green signals as high polysomy.

Results

Of the 90 patients, 30 with a score of 0/1+ (negative) by IHC were FISH negative for HER-2/*neu* gene amplification (ratio between 0.6-1.6) (Fig. 1 A, B, Fig. 2 A, B). Of the 28 patients with the score of 2+ by IHC (Fig. 2 C), 20 were FISH positive for HER-2/*neu* gene amplification (>2.2), three were FISH negative and five patients showed equivocal (1.8-2.2) results by FISH (Fig. 1C). These five cases were retested for IHC and FISH on different paraffin embedded tissue blocks, where all five patients were found eligible for trastuzumab-based therapy. Twenty five patients with the score of 3+ by IHC (Fig. 2 D) were FISH positive for HER-2/*neu* gene amplification with the ratio of >3.9 (>2.2) (Fig. 1 D). Seven cases with the score of 3+ by IHC were FISH negative for HER-2/*neu* gene amplification (>2.2). These cases showed polysomy of chromosome number 17 (spec green signals > 4) which could have been the reason for IHC 3+ (Fig. 3). The association of HER-2/*neu* status in these 90 patients with histological grade of tumour and estrogen and progesterone receptor status is shown in the Table.

Discussion

The HER-2/*neu* gene is located on the long arm of chromosome 17 (17q12-21.32). It encodes p185 oncoprotein which is a receptor tyrosine kinase that can be associated with multiple signal transduction pathways. It has been found to be overexpressed in many types of human malignancies, notably breast,

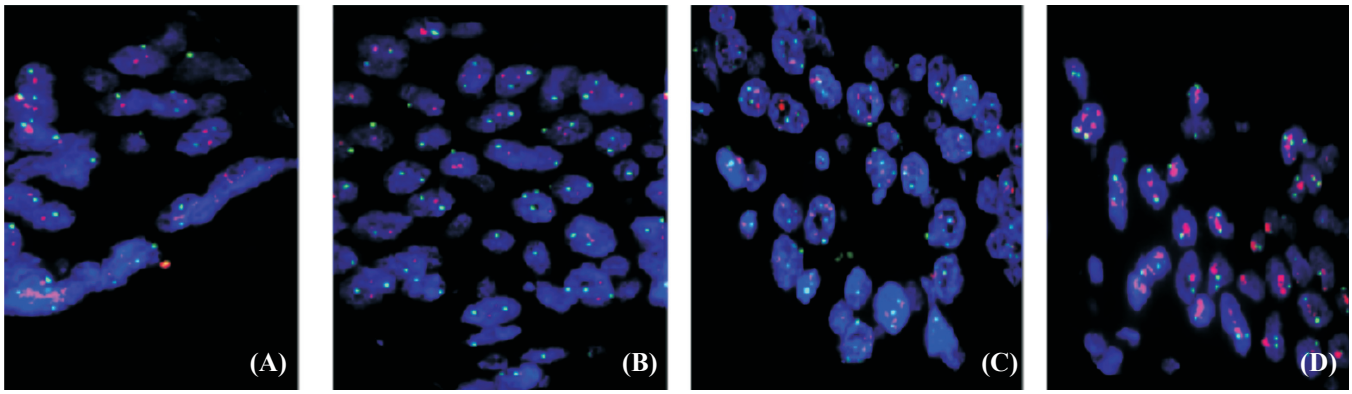

Fig. 1. Fluorescence *in situ* hybridization (FISH) images showing negative, equivocal and positive results for HER-2/*neu* gene amplification. **(A)** Ratio of HER-2/*neu* (orange) signal to centromere 17 (green) signal 0.6-1.1 negative by FISH, **(B)** Ratio 1.6 negative by FISH, **(C)** Ratio 1.8-2.2, equivocal by FISH, **(D)** Ratio 3.9 positive by FISH.

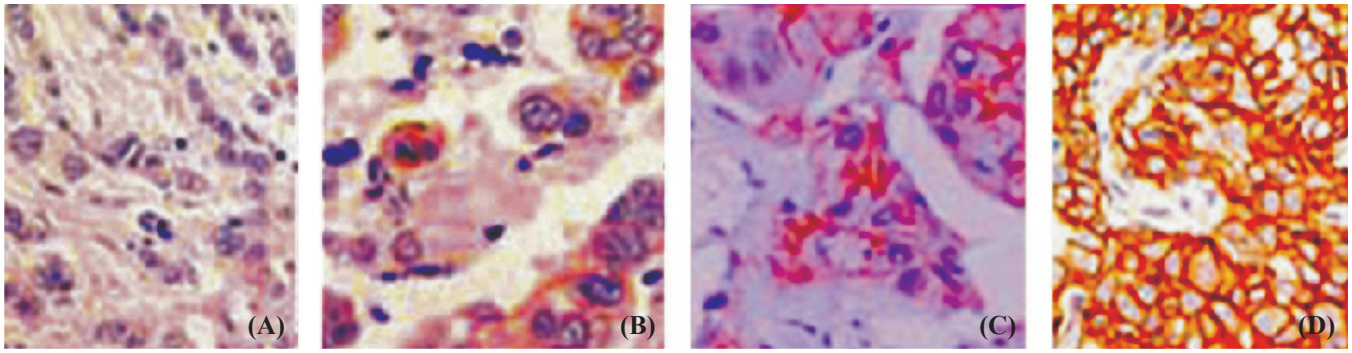

Fig. 2. Immunohistochemical (IHC) analysis results showing immunostaining grades image with rations 0 to 1+ are negative for HER-2/*neu* membrane staining and 2+ are equivocal and 3+ are positive for HER-2/*neu* membrane staining. **(A)** Score 0, normal by IHC, **(B)** Score 1+ normal by IHC, **(C)** Score 2+ equivocal by IHC, (D) Score 3+ abnormal.

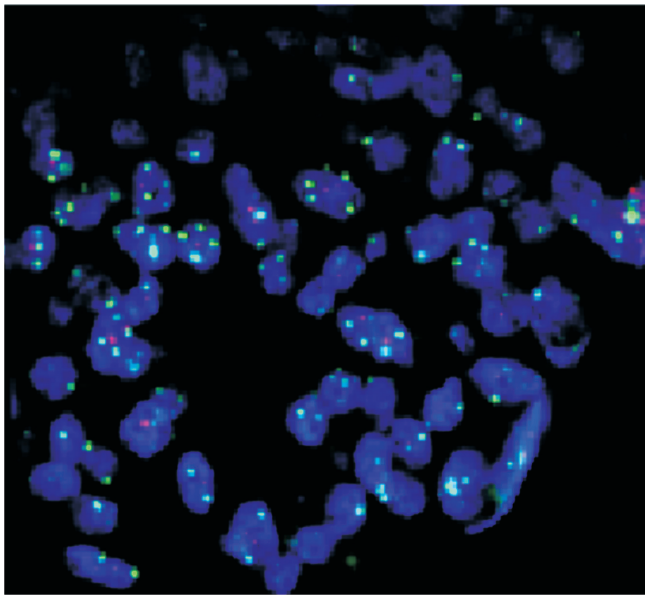

Fig. 3. Polysomy 17 showing CEP 17 spec green signals= 4 by FISH.

ovarian, gastric, pancreatic, prostatic, colorectal, cancers of the female genital tract and lung cancer. HER-2/neu, also known as erbB-2 oncoprotein is overexpressed in 25 to 30 per cent of breast cancers[14]. HER-2/neu overexpression in patients with breast cancer and positive lymph nodes is linked to poor prognosis with a reduced disease-free interval and shortened survival time, and similar linkage may exist in node-negative cases[14-16]. HER-2/*neu* gene expression level seems to be a significant predictor for response to some therapeutic agents[14,17]. Trastuzumab (Herceptin), a humanized monoclonal anti–HER-2/*neu* antibody, was approved by the US Food and Drug Administration as an adjuvant therapeutic agent for patients with metastatic breast cancer overexpressing HER-2/*neu* protein[18,19-22]. As a result, evaluation of HER-2/*neu* status has become pivotal in determining patient's eligibility for trastuzumab treatment.

Table. Association of HER-2/*neu* status with clinical, estrogen and progesterone receptor (ER, PR) status and histological parameters

Parameter	HER-2/*neu* gene amplification by FISH			
	Positive	Negative	Equivocal	Polysomy for HER-2/*neu* gene amplification
1. Age (yr):				
<40 (n=57)	28	22	3	4
>40 (n=33)	17	11	2	3
2. Histologic grade:				
I (n=2)	0	02	0	0
II (n=20)	5	12	1	2
III (n=68)	40	19	4	5
3. Estrogene receptor status:				
Positive (n=31)	17	12	1	1
Negative (n=59)	28	21	4	6
4. Progesterone receptor status:				
Positive (n=24)	05	19	1	2
Negative (n=66)	40	14	4	5

In clinical laboratories, HER-2/*neu* status is usually assessed in formalin-fixed and paraffin-embedded specimens using either IHC or FISH. Wang *et al*[22] achieved a high concordance rate of 98 per cent with two FISH assays, Inform (Ventana Medical Systems, Tucson, AZ) and PathVysion (Vysis). All IHC negative cases and nearly all IHC low positive (1+) cases showed no gene amplification, whereas most IHC high positive (3+) cases had gene amplification. However, the IHC medium positive (2+) cases demonstrated significant discordance with the FISH assay, *i.e.*, some showed HER-2/*neu* gene amplification and others did not. Similar discordant results were seen in our patients; 30 patients with the score of 0/1+ by IHC were FISH negative for HER-2/*neu* gene amplification (ratio between 0.6-1.6), among 28 patients with the score of 2+ by IHC, 20 were FISH positive, three were FISH negative and five showed equivocal (1.8-2.2) results by FISH. The most appropriate *HER-2* status for such patients falls in this gray zone (now defined as a FISH ratio of 1.8-2.2) and remains unclear. To address this, the new American Society of Clinical Oncology/ College of American Pathologists (ASCO/CAP) HER2 guidelines[23] have added equivocal categories for FISH results, similar to those for immunohistochemical analysis, in the hope that this will prompt further study to better define the potential benefit from HER2-directed therapy for patients with FISH results that fall within this equivocal or borderline category[24,25]. In our study, the five cases retested by IHC and FISH on different paraffin embedded tissue blocks, were positive for HER-2/*neu* gene amplification and were eligible for trastuzumab-based therapy. The reason could be due to the heterogeneous nature of the tumour showing variation in results. Thus, FISH equivocal cases can be rechecked on different tissue block of the same patient.

In our study, younger breast cancer patients were reported to have higher frequency of *HER-2*

gene amplification. Panjwani *et al*[26] showed positive correlation of grade III with HER-2/*neu* amplification which was concordant with existing literature. An inverse association was noted between hormone receptor status and *HER-2* gene amplification in the present study. The reason could be the complex signaling between ER and other growth factor signaling pathways in breast cancer cells[26]. In our study, 18 per cent cases showed both expression of ER and *HER2* amplification. *HER2* amplification in these tumours is reported to be associated with resistance to tamoxifen therapy[25]. It is postulated that in these tumours, tamoxifen functions as an estrogen agonist to enhance growth in breast cancer cells which express high levels of HER2 and estrogen receptor co-activator resulting in *de novo* resistance for tamoxifen[26].

Seven cases with the score of 3+ by IHC were FISH negative for gene amplification (>2.2). Earlier studies have shown similar results[23,27-29]. Although there is good correlation between *HER-2* gene amplification and protein overexpression, many studies[23] also have shown that 3 to 15 per cent of breast carcinomas over-express the HER-2 protein without gene amplification and a small subset of breast carcinomas amplify the HER-2 gene without overexpression. Various theories have been proposed to account for these discrepancies. Increased receptor expression in breast cancer without genetic alterations has been reported in approximately 10 per cent of cases, probably caused by transcriptional or post-translational activation. Other proposed explanations for the phenomenon of overexpression without amplification include artifactual high sensitivity of immunohistochemical assays, single copy overexpression of the *HER-2* gene at the messenger RNA (mRNA) transcription level, or gene amplification below the level of detection of FISH assays[30,31]. The presence of chromosome 17 polysomy is not uncommon in breast carcinomas and has been suggested to account for overexpression without amplification[23]. We found that average chromosome 17 copy number when compared to average HER-2/*neu* copy number, was significantly higher in FISH interpreted as polysomy of chromosome 17, and this could be falsely interpreted as HER2/*neu* overexpression by IHC analysis. This would be reasonable to examine whether these IHC 3+/FISH-negative tumours with polysomy 17 are biologically distinct from other FISH-negative tumours and more similar to tumours with conventional HER-2/*neu* amplification, especially in terms of their response to Herceptin-based therapy. Hence, all breast cancer cases with an HER2 immunohistochemical score of 2+ should be reflex tested for HER*2/neu* gene amplification using FISH.

The discussion on the best method to determine HER-2/*neu* status in these samples continues, with the FISH method gaining popularity due to the recent evidence that it, in comparison with IHC, may more accurately predict clinical responses to trastuzumab-based therapies[28]. Investigation of HER-2/*neu* amplification along with frequent genetic changes would be valuable in determining prognostic factors. The equivocal cases evaluated by IHC and FISH should be interpreted with caution and such patients should be monitored on follow up. The current study found that a combined approach using both IHC and FISH methodologies can optimize HER-2/*neu* testing on breast carcinomas. There is also a need for establishing a clinically validated cut-off value for HER-2/*neu* FISH amplification against which IHC may be further compared and calibrated. This would potentially allow for more accurate and clinically meaningful HER-2/*neu* testing in the future.

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