

RESEARCH COMMUNICATION

Immunohistochemical (IHC) HER-2/neu and Fluorescent-In-Situ Hybridization (FISH) Gene Amplification of Breast Cancer in Indian Women

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Abstract

Background: The concordance rate between immunohistochemical (IHC) and fluorescence in situ hybridization (FISH) results for HER2/neu according to clinical performance is controversial. The present prospective study was therefore conducted in Indian breast cancer patients. **Methods:** Fifty cases (n=50) of invasive duct cancer of breast tested for HER-2/neu by IHC and scored as 0, 1+, 2+ and 3+ by pathologists were further analyzed by FISH using a commercially available double-color probe, and the findings compared. **Results:** A total concordance of 82.0% was observed with a Kappa coefficient of 0.640 (P < 0.001). A high discordance was observed in 30.0% of the patients with IHC 2+, 7.1% in IHC 3+, 19.2% overall in IHC 0 and 1+. **Conclusion:** IHC can be used firstly to screen the HER-2/neu status, and FISH can be used as a supplementary role to IHC and 2+ and some negative cases. And only those cases with HER-2/neu status of IHC 3+ or FISH positive should be treated with Herceptin (Trastuzumab).

Keywords: Breast cancer - HER-2/neu - FISH - IHC - Herceptin (Trastuzumab)

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Introduction

Breast cancer is one of the most common malignancies in the world. According to the global cancer statistics, Europe and America have the high incidence and mortality (Parkin et al., 2005). The incidence of breast cancer is 20 per 100,000 populations, and the incidence is growing (Zheng et al., 2001).

Research has shown that about 20% - 30% of the breast cancer patients have HER-2/neu amplification or overexpression, which is associated with a more aggressive phenotype and decreased survival (Paterson et al., 1991; Seshadri et al., 1993; Press et al., 1993; 1997; Ross and Fletcher, 1999). The benefit of humanized anti-HER-2/neu monoclonal antibody trastuzumab (Herceptin) in HER-2/neu -positive breast cancers has been well documented as noted by prolonged survival (Dean-Colomb and Esteva, 2008). But this therapy is effective only if the detection of HER-2/neu status is accurate. There are several methods available to detect the HER-2/neu status like polymerase chain reaction (PCR), immunohistochemistry (IHC), fluorescence-in-situ hybridization (FISH), Chromogenic in situ hybridization (CISH) (Tanner et al., 2000; Sáez et al., 2006). Protein overexpression detected by IHC or amplification of HER-2/neu gene analyzed by FISH are the two main methods used to detect HER-2/neu status

in clinical practice. FISH is considered as a gold standard because of its sensitivity and specificity. But FISH has disadvantages as it requires a modern and expensive fluorescence microscope equipped with multi-band-pass fluorescence filters, and the fluorescence fades so quickly that it could not provide a permanent record (Sáez et al., 2006). Compared with FISH, IHC is widely used in China as it is cheaper and convenient to operate and conserve; the morphology is clear. Comparative studies of IHC and FISH have generally shown a high concordance rate (Reed et al., 2000). But protein overexpression may be found without gene amplification or gene amplification can be found in negative IHC (Pauletti et al., 2000). Research has documented that the discordance rate between HER-2/neu by FISH and IHC is high in all four IHC scores (0, 1+, 2+, 3+), and a FISH-alone screening strategy has been alternatively suggested (Tubbs et al., 2001). Our objective was to perform a prospective study in our own local setting and record the concordance between IHC and FISH in 50 cases of invasive duct cancer of breast.

Materials and Methods

Tumor collection and fixation protocol

Surgically removed breast cancer tissues were collected from Grant Medical College and Sir J J Group

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of Hospitals, Mumbai, India taking tissue samples of breast cancer patients (n=50). Expression of HER-2/neu IHC and FISH were analyzed in specimens of infiltrating duct breast cancer tissue of Indian women during radical mastectomy and lumpectomy. IHC study was performed with instructions of American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human Epidermal Growth Factor Receptor-2 testing in breast cancer (Wolff et al., 2007).

Immunohistochemistry

Tissue sample were fixed in fixative 10% neutral buffered formalin (fixative) for 12- 24 hours. Processing tissue sample in auto processor, embedding the tissue with paraffin wax on embedding station, cutting of paraffin blocks by microtome 4 μ thickness sections were dried overnight at 37°C. Prior to antibody staining, the slides were pre-treated with microwave irradiation to unmask binding epitopes. After blocking of endogenous peroxides activity with a 3% solution of hydrogen peroxide in methanol for 30 minutes, slides were immersed in 200 ml of 10 mM citric acid (pH 6.0) for 5 minutes on power (100 W) after that 4 cycle 5 minute each on power (50W). After topping up of the buffer with distilled water, this step was repeated. The slides were then left to stand for 10 minutes in buffer at room temperature before being washed thoroughly in tap water.

After three washes in tris-buffered saline (TBS), the slides were incubated with a 1:25 dilution of mouse anti-HER-2/neu monoclonal primary antibody (Clone: CB11; NCL-L-CB11; Visionbiosystems Asia Pacific) in TBS for 1 hour at room temperature. After three more washes in TBS, than secondary antibody (K0355; *DakoCytomation, Denmark*) Biotinylated Goat Antibody (LINK) to Mouse / Rabbit Immunoglobulin, Dilute antibody (1:100) in TBS was applied for 1 hour at room temperature. After an additional three washes. Than Streptavidin - Biotin / HRP; Horse Radish Peroxidase Complex (Enzyme Label) (K0355; *DakoCytomation, Denmark*) Dilute antibody (1:50) in TBS was applied for 1 hour at room temperature. After an additional three washes, the staining was visualized by adding diaminobenzidine (DAB kit; K3467; *DakoCytomation, Denmark*) for 5 minutes at room temperature. The slides were washed well in tap water and counterstained with Harris's haematoxylin for 10 seconds to 1 minute and then dehydrated, cleared, and mounted in Distrene Plasticiser Xylene (DPX) (Dolan and Snover, 2005). Positive and negative controls were performed with each batch of slides. Surgical specimens from the same patient were stained on the same run.

Fluorescent -In-Situ Hybridization

FISH for detection of HER-2/neu gene amplification in formalin-fixed, paraffin-embedded breast cancer tissue specimens was performed using the PathVysion HER-2/neu probe kit (LSI; Locus Specific Identifiers HER-2/neu, Spectrum Orange / CEP 17; Centromeric Enumeration Probe 17 Spectrum Green *Vysis Inc, Downers Grove, IL*), 17q11.2-q12 (labeled with Spectrum Orange) covering the whole Her-2 gene and the control, centromeric chromosome 17p11.1-q11.1 (labeled with

Spectrum Green), was used for the FISH analysis. In brief, the sections were baked overnight at 56°C, and the invasive breast cancer components were located on a corresponding H & E stained section. Unstained sections were deparaffinized in CitriSolv (*Vysis Inc, Downers Grove, IL*), dehydrated in 100% ethanol, and air-dried. Slides were then subjected to protease digestion for 60 minutes, denatured, and hybridized with pre-warmed probes (HER-2/neu, / CEP17; Spectrum Green probe; *Vysis*) overnight at 37°C. They were then washed with post-hybridization wash buffer at 72°C and counterstained with DAPI, (4', 6-Di Amidino-2-Phenyl Indole) mounted, and stored in dark before signal enumeration. Slides were first scanned at low power using a DAPI filter to identify areas of optimal tissue digestion and non-overlapping nuclei. The number of chromosome 17 signals, HER-2/neu signals, and the number of tumor nuclei scored were recorded for each case. Cases were interpreted as amplified when the ratio of HER-2/neu chromosome 17 signals was equal or greater than 2.0.

The entire stained slide was scanned for immunostaining evaluation by light microscope. The image collection and microphotographs were taken by the Axio Imager. M1 Microscope with the AxioVision software; *Carl Zeiss Microscopy Germany*. Check the slide under 10X objective to confirm that the cells are still attached to the slide. Finally focus under X400 objective magnification. All images were taken under X400 objective magnification without oil immersion lens. All images were processing with AxioVision software.

Analysis of FISH was on an Olympus AX70 fluorescence microscope with appropriate filters using the Cytovision software. Check the slide under 10X objective to confirm that the cells are still attached to the slide. Check for blue fluorescence of DAPI, (4', 6-Di Amidino-2-Phenyl Indole) indicating the presence of nuclei. Check the hybridization of the probe under 100X objective using appropriate filters. Focus up and down to find all of the signals present in the nucleus. Count two signals that are the same size and separated by a distance equal or less than the diameter of the signal as one signal. Do not score nuclei with no signals or with signals of one color only. Record the counts in a two-way table, which is saved under the appropriate accession number. Enumerate and analyze 60 nuclei for the signal pattern strictly following the above criteria. Criteria for analysis and enumeration Count only DAPI stained cells with defined nuclear borders. Do not count overlapping cells. Two signals of the same fluorochrome that are in close proximity but more than a diameter apart from each other, approximating the same size but not connected by a visible link are counted as two signals.

Scoring Methods

IHC and FISH gene amplification scoring of HER-2/neu is as follows (after ???): True HER-2/neu positivity is given by crisp brown colored membrane staining present, in at least 30% of the Invasive tumor score 3 that is two steps higher than HER-2/neu expression in surrounding benign breast parenchyma.

Tissue HER-2/neu receptor over expression and/or

Table 1. Criteria for

Score HER-2/neu Protein Staining Pattern		
Over Expression Assessment		
0	Negative	No staining is seen, or staining is seen in less than 10% of tumor cells.
1+	Negative	A faint / barely perceptible membrane staining is detected in more than 10% of tumor cells but the cells are only stained in part of the membrane.
2+	Borderline or Weakly Positive	Weak to moderate complete membrane staining is seen in more than 10% of tumor cells.
3+	Strongly Positive	Strong complete membrane staining is seen in more than 30% of tumor cells.

Table 2. Criteria for FISH Results

Result	HER-2/neu FISH (Two Approaches)	
	IHC Results	
Positive	3+	<6 HER-2/neu copies per cell or HER-2/neu: ch 17 ratio of 2.2 to 1
Negative	0+, 1+	<4 HER-2/neu copies per cell or HER-2/neu: ch 17 ratio <1.8 to 1
Equivocal or 2+ Borderline		4 - 5 HER-2/neu copies or 1.8 – 2.2 HER-2/neu: ch 17 ratio

HER-2/neu gene amplification is essential for treatment with anti HER-2/neu monoclonal antibody (trastuzumab) i.e. Herceptin, which has significant clinical benefit in patients with metastatic breast cancer. Score 0, 1 are unequivocally negative with no further intervention recommended shown in Table 1.

For a borderline / weakly positive score of 2+ on IHC, however; it is recommended that this be followed by a FISH assay, where a FISH ratio > 2.0 is pathognomonic of HER-2/neu gene amplification with likely benefit to be had from treatment with Herceptin. IHC and FISH are the two testing methods recommended in the ASCO/CAP HER-2/neu testing guideline, which detail how the tests should be performed and what constitutes HER-2/neu positive, negative and equivocal or borderline results. The IHC assay measures the amount of HER-2/neu protein expressed in cancer cells, while the FISH assay measures the total number of copies of the HER-2/neu gene in a tumor cell or the ratio of HER-2/neu to chromosome 17 copies.

The number of LSI HER-2/neu; (Locus Specific Identifiers HER-2/neu) and CEP 17 (Centromeric Enumeration Probe 17) signals per nucleus are recorded in a two-way table. Results on enumeration of 60 interphase nuclei from tumor cells per target are reported as the ratio of average HER-2/neu copy number to that of CEP 17. Clinical study found that specimens with amplification showed a LSI HER-2/neu: CEP 17 signal ratio of ≥2.0; normal specimens showed a ratio of <2.0. Result at or near the cutoff point (1.8 - 2.2) shown in Table 2.

Analysis of FISH was on an Olympus AX70 fluorescence microscope with appropriate filters using the Cytovision software. Check the slide under 10X objective to confirm that the cells are still attached to the slide. Check for blue fluorescence of DAPI, (4', 6-Di Amidino-

2-Phenyl Indole) indicating the presence of nuclei. Check the hybridization of the probe under 100X objective using appropriate filters. Focus up and down to find all of the signals present in the nucleus. Count two signals that are the same size and separated by a distance equal or less than the diameter of the signal as one signal. Do not score nuclei with no signals or with signals of one color only. Record the counts in a two-way table, which is saved under the appropriate accession number. Enumerate and analyze 60 nuclei for the signal pattern strictly following the above criteria. Criteria for analysis and enumeration Count only DAPI stained cells with defined nuclear borders. Do not count overlapping cells. Two signals of the same fluorochrome that are in close proximity but more than a diameter apart from each other, approximating the same size but not connected by a visible link are counted as two signals.

Statistical Analysis

Calculate mean, χ^2 -test, P-value, concordance, discordance and a Kappa coefficient. The computing was carried out using the SPSS-16 procedure (SPSS Analytical Software Inc, Chicago, IL). Statistical significance was calculated by the likelihood ratio, Wald, score, and chi-square test and the worst test result was taken. Likelihood ratio test served to check for significant differences and the worst level of significance.

Results

IHC and FISH staining of 50 cases (n=50) of infiltrating duct cancer of the breast in Indian women. The entire IHC slide was scanned for immunostaining evaluation by light microscope. Tissue sections exhibiting membrane staining over expression positive immunoreactivity for HER-2/neu in tumor cells were identified shown in Figure 1a. Negative membrane immunostaining shown in Figure 1b.

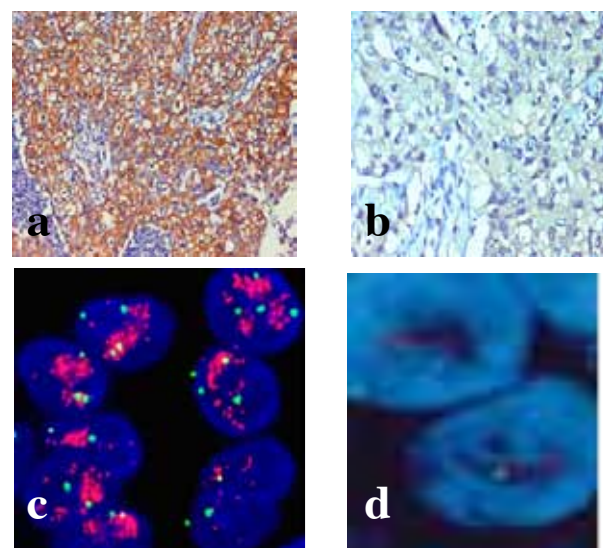


Figure 1. Results for Infiltrating Duct Breast Cancer. a) Membrane positive staining for IHC- HER-2/neu receptor; b) Negative membrane staining for HER-2/neu receptor; c) Tumor cells showing of FISH- HER-2/neu gene amplification; d) Tumor cells showing negative FISH-HER-2/neu gene amplification

Table 2. Comparison of the Results of IHC and FISH

IHC Scoring	HER-2/neu FISH Amplified	HER-2/neu FISH Non-Amplified	Concordance by IHC	Discordance by IHC
0 and 1+ (n = 26)	5	21	(21/26) 80.8%	(5/26) 19.2%
2+ (n = 10)	7	3	(7/10) 70.0%	(3/10) 30.0%
3+ (n = 14)	13	1	(13/14) 92.9%	(1/14) 7.1%

The cells were analyzed for FISH on an Olympus AX70 fluorescence microscope with appropriate filters, using the Cytovision software. Tumor cells showing of HER-2/neu gene amplification in infiltrating duct cancer of breast showed in Figure 1c and tumor cells showing negative HER-2/neu gene amplification shown in Figure 1d.

50 specimens of infiltrating duct cancer of Indian women in our study, varying tumor grades and clinical stages, were classified as IHC 0, 17 were classified as IHC 1+, 10 were classified as IHC 2+, and 14 were classified as IHC 3+. Five of the IHC 0 and 1+ cases, seven of the 10 IHC 2+ cases and 13 of the 14 IHC 3+ cases were found to be HER-2/neu FISH positive. They had a total concordance of 82% and a Kappa coefficient of 0.640 ($P < 0.001$), which was defined as IHC 2+ / 3+ and HER-2/neu FISH positive, or IHC 0 / 1+ and HER-2/neu FISH negative. Discordance was defined as a discrepancy between the IHC and HER-2/neu FISH, including the following two conditions:

- IHC 2+ or 3+ but HER-2/neu FISH negative;
- IHC 0 or 1+ but HER-2/neu FISH positive.

For example, the discordance rate according to IHC 0 and 1+ was defined as the number of discrepant IHC 0 and 1+ cases divided by the total number of IHC 0 and 1+ cases and was 19.2% (5 / 26). Following the same way of counting, the discordance rate according to IHC 2+ was 30% (3/10), IHC 3+ was 7.1% (1 / 14). The overall discordance rate by IHC was therefore 18% (9 / 50) shown in Table 2.

Discussion

Reliable laboratory data in evaluating HER-2/neu status is essential, because the treatment is beneficial for advanced breast cancer and can avoid potential cardiotoxic effects in women not showing amplification or overexpression (Keefe, 2002). HER-2/neu status studied at the levels of DNA using FISH and protein using IHC are the two most accessible and feasible methods used in clinical diagnosis, and certain kits or antibodies are approved by the FDA (U.S. Food and Drug Administration). IHC is easy to perform and relatively cheap, and is predominantly used to evaluate HER-2/neu status. However, a wide range of sensitivity and specificity has been observed among various commercially available antibodies (Bemtp et al., 2005). As an alternative, FISH is also recognized as a modality in cases with an equivocal IHC status with higher sensitivity and specificity. Considering FISH as a gold standard, research reports that positive FISH results could be found in 91.7%, 23.2%, 7.4% and 4.1% in cases with IHC respectively diagnosed as 3+, 2+, 1+ and 0 (Owens et al., 2004). In addition, some clinical trial have shown that amplification by FISH is more predictive of response to trastuzumab than IHC (Pauletti et al., 2000; Dybdal et al., 2005; Mass et al., 2005). Our objective in

this study was not to try and compare the sensitivity and specificity of these two tests (IHC and FISH). This study aimed to investigate the concordance and discordance rates between IHC and HER-2/neu FISH. Some similar studies adopting a similar strategy have been reported. Dolan et al found that the concordance between the IHC and FISH scores (defined as cases that were IHC negative/FISH non amplified or IHC positive/ FISH amplified) was found in 35 cases (27.1%) and discordance in 94 cases (72.9%) (Dolan and Snover, 2005). Lan et al used FISH to ascertain the prevalence of erb-b2 gene amplification in 221 cases of breast cancer specimens read as 2+ in IHC analysis, and found 96 (44.4%) cases were detected to be erb-b2 amplified (Lan et al., 2005). Kuo et al compared FISH and IHC in breast cancer patients and found that the discordance rates by IHC were high (46.7% in IHC 2+, 16.7% in IHC 3+, 30.3% overall in IHC 2+ or 3+) (Kuo et al., 2007). All these researches indicated that the concordance between IHC and FISH is still controversial. There are some factors leading to false IHC test results, including variability in tissue fixation and processing, variable sensitivity and specificity of commercially available antibodies, and differences in scoring criteria with considerable inter observer variability in interpretation of results (Jacobs et al., 2000).

Our study is trying to answer a simpler question about the concordance between IHC and FISH, our concordance in the cases of IHC 3+ is similar with theirs, but there are some differences in the cases of IHC 0, 1+, 2+. The concordance in this study of IHC 0 and 1+ is lower, and IHC 2+ is higher than theirs. This may due to the different sensitivity and specificity of the antibodies and probe used in this study. The different sensitivity and specificity of the antibodies and probe need to be further researched.

In conclusion, in order to improve the therapeutic effect of Herceptin in HER-2/neu - positive breast cancers, the current algorithm using FISH as a supplementary role to IHC 2+ need to be modified according to this study in our setting. The IHC can be used firstly to screen the HER-2/neu status, and FISH can be used as a supplementary role to detect IHC and 2+ and some negative cases, especially those with a high tumor grades. And only those cases with HER-2/neu status of IHC 3+ or FISH positive are proposed to be treated with Herceptin (Trastuzumab).

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