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Original Article

Pathology Section

HER2/*neu* Testing In 432 Consecutive Breast Cancer Cases using FISH and IHC - A Comparative Study

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ABSTRACT

Introduction: The development of trastuzumab, a HER-2/*neu* targeted monoclonal antibody resulted in significant improvements in clinical response and survival in HER-2/*neu* gene amplified group of patients. Thus, accurate assessment of HER-2/*neu* status becomes critical. Fluorescence In Situ Hybridization (FISH) and Immunohistochemistry (IHC) are the most commonly used methods for this purpose and specific recommendations exist with regard to the concordance to be observed between the two tests.

Aim: Here, we report and evaluate the concordance rate between FISH and IHC for HER-2/*neu* status in breast cancer specimens.

Materials and Methods: Archival paraffin blocks of tumour tissue from 450 patients of breast cancer were analyzed for Her-2/*neu* status using FISH and IHC.

Results: There was a highly significant concordance between the results of FISH and IHC assays in HER-2/*neu* status assessment in invasive breast cancer cases. There were inverse associations between the expression of Oestrogen Receptors/ Progesterone Receptors (ER/PR) and HER-2/*neu* amplification.

Conclusion: Although, IHC gave significant concordant results with FISH in determining HER-2/neu status, its subjective grading system precludes its use as a gold standard. FISH should always be used to determine true gene amplification when the IHC results are equivocal.

Keywords: Concordance, Molecular diagnostics, Theranostics

INTRODUCTION

HER-2/*neu*, a member of the human epidermal growth factor receptor, family of tyrosine kinases, is involved in critical signaling pathways that control cell proliferation and survival [1]. Aberrant function of this receptor, due to acquired genetic defects that result in gene duplication and over-expression of the protein, has been implicated in a variety of cancers [2]. In breast cancer, HER-2/*neu* gene amplification and protein over-expression has been reported in 20-25% of cases and was traditionally associated with poor prognosis due to an aggressive tumour phenotype, increased metastasis and poor survival [3]. However, this underwent a volte-face with the advent of trastuzumab, a HER-2/*neu* targeted humanized monoclonal antibody therapy which resulted in significant improvements in clinical response and survival in these patients [4]. In addition, HER-2/*neu* status has been predictive marker for response to other anti cancer agents like a better response to anthracyclines [5], resistance to cyclophosphamide [6] and tamoxifen [7]. Also, FDA approved targeted therapy molecules such as lapatinib and pertuzumab have offered an effective personalized treatment modality for patients having the HER-2/*neu* gene amplification and expression [8]. Thus, accurate identification of HER-2/*neu* positive invasive breast carcinoma patients becomes an important prerequisite for appropriate treatment and follow up regimens.

HER-2/*neu* status can be assessed at various cellular levels using different laboratory techniques; at the protein level by IHC and ELISA, at the RNA level by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) and microarray, at the DNA level by in situ hybridization techniques utilizing fluorescence (FISH) or chromogens (CISH) [9]. IHC and FISH are FDA approved and the most commonly followed methods [10]. IHC, a semi-quantitative analysis of HER-2 protein expression, is quick, easy and economical but is more susceptible to discrepancies in test results due to variations in laboratory parameters [11]. FISH, a quantitative analysis of HER-2/*neu* gene copy number, requires more time and expense but has more reliability due to its quantitative nature [12,13]. The polemic about the most suitable test for HER-2/*neu* status determination is ongoing with different groups espousing different views regarding the gold standard to be followed [14].

American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines consider FISH and IHC as equivalent methods and recommend reflex testing in equivocal cases of either assay. Also, the panel recommends concordance levels of at least 90% and 95% amongst the positive and negative reports respectively of each test [15,16]. To this end, laboratories reporting on the HER-2/*neu* status in patient samples must routinely check the concordance between the various molecular tests that they employ. In this paper, we report the results of both IHC and FISH done on 432 cases of breast cancer at an Indian tertiary cancer care centre. In addition, all samples were tested for ER/PR expression. Concordance rates between IHC and FISH were evaluated. Further, correlation of receptor expression with clinical factors like age was also considered.

MATERIALS AND METHODS

Patient Samples

Formalin fixed paraffin embedded blocks of tumour tissue from 450 patients with a histologically confirmed diagnosis of breast cancer received at the Division of Molecular Pathology, Triesta Reference Laboratory, HealthCare Global Enterprises, Bengaluru, Karnataka, India, between August 2007–November 2013 were used in the study. The inclusion criteria was that the histological type of the specimen should have been Invasive Ductal Carcinoma-Not Otherwise Specified (IDC-NOS). All special histological types were excluded from the study. The study was approved by the institutional review board with informed consent being obtained from all patients. The demographic data of each case was noted.

IHC Assays

Sections of suitable thickness (3 μm) were cut from blocks having adequate well preserved invasive cancer and placed on acid pretreated poly-L-lysine-coated slides to incubate overnight at 56ºC. Sections were de-paraffinized via two changes of xylene and further rehydrated through graded alcohols to distilled water. After blocking endogenous peroxidase activity with 3% hydrogen peroxide in methanol, antigen retrieval was achieved by heating the slides in 10 mmol/l citrate buffer (pH 6) using a water bath. Rabbit monoclonal anti HER-2/*neu* primary antibody (Dako, Glostrup, Denmark) was applied for 60 min at 1:800 dilution. The Envision Kit (Dako) was used for application of the secondary antibody, signals were developed with Diaminobenzidine (DAB) followed by light nuclear counter staining with haematoxylin. Each test batch was run with a known positive and negative control. To evaluate the immunostaining for the ERBB2 antibody, ASCO/CAP guidelines [16] were considered. Expression was recorded in invasive cancer cells as: score 0 = no staining observed; score 1+ = weak incomplete moderate membrane staining in greater than 10% of tumour cells; score 2+ = weak to moderate intensity circumferential membrane staining in greater than 10% of tumour cells; score 3+ = strong complete membrane staining in greater than 30% of tumour cells. For ER/PR detection, pre staining and peroxidase block processes were like the one used for HER-2/*neu* staining. Heat Induced Epitope Retrieval (HIER) was achieved by heating the slides in EDTA buffer (pH 8) for 25 minutes at 95ºC using an automated EZ Biogenex microwave. Primary antibody clones used for ER and PR were DAKO 1D5 (1:400) and Biogenex PR 88 (1:600) respectively. Signals were developed as described earlier. Interpretation of nuclear intensity and proportion of invasive cancer cells that displayed staining was done as per ER/PR reporting guidelines [17]. Appropriate mandatory external tissue controls and if present internal controls were used.

FISH Assay

PathVysion Test Kits (Abbott Laboratories, Abbott Park, IL, USA) were used for FISH. This kit comprises of two probes; a red Locus Specific Identifier (LSI) for HER-2/*neu* and a green Centromere Enumeration Probe (CEP) [17]. The test was performed on paraffin sections mounted on acid treated double poly-L-lysine coated glass slides as per the manufacturer's instructions. Slides were scored immediately using an upright fluorescence microscope equipped with appropriate excitation and emission filters to allow visualization of the signals. Interpretation was independently done by two pathologists. The results were then compared and a consensus score recorded. In case of variable results, the assay was repeated. The fields containing invasive tumour component with non-overlapping tumour nuclei were chosen for interpretation. A minimum of 60 tumour nuclei, showing at least one green and one red signal, were counted for each case. The ratio of the HER-2 (red) to CEP 17 (green) signals for the 60 tumour nuclei was calculated. Other features like polysomy 17 were also noted. Fields showing excess background signals or auto-fluorescence masking the nuclear signals were not evaluated. As proposed by the ASCO/CAP guidelines (2007), a HER-2/CEP17 signal ratio of less than 1.8 was considered HER-2 negative; a HER-2/CEP ratio between 1.8 and 2.2 was considered HER-2 equivocal and a HER-2/CEP17 ratio more than 2.2 was considered HER-2 positive. Polysomy 17 was defined as the presence of 3 or more copies of CEP 17 in more than 10% of the tumour nuclei [18]. Adjacent normal tissue was used as an internal negative control. A recounting by a third pathologist was done for the equivocal cases. Normal and amplified control slides were run simultaneously with the test cases. The slides were then stored in the dark at -20ºC.

STATISTICAL ANALYSIS

Data analysis was performed using SPSS 11.5 software. The concordance between IHC and FISH was evaluated by calculating the percent agreements [19] and cohen's kappa coefficient. In addition, the specificity, sensitivity, positive predictive value and negative predictive value of IHC were calculated. Contingency tables were analysed using the Fisher-exact test to detect significant associations between different variables. A p value<0.05 was considered significant.

RESULTS

This retrospective study reports on a total of 432 invasive primary breast cancer cases which were analyzed for HER-2/*neu* gene amplification (FISH), HER-2 protein expression (IHC) as well as ER and PR expression. Although there were 450 cases in total, FISH assay failed in 18 cases (18/450; 4.00%) due to various reasons and were excluded from the study.

Clinical parameters

All except six (6/432; 1.38%) were female (426/432; 98.62%) patients. The median age of the patients was 53 years (range: 25–85). 170 (39.35%) patients were ≤ 50 years while 262 (60.65%) were >50 years in age. There was no significant difference between the two age groups with respect to HER-2/*neu* status by FISH/IHC as well as ER/PR status.

HER-2 status

FISH was done to ascertain HER-2/*neu* gene amplification. HER-2/*neu* was non-amplified in more than half of our cases (223/432, 51.62%) whereas it was amplified in 46.29% cases (200/432). Nine cases were reported as equivocal in FISH (9/432; 2.08%) and were excluded from statistical analysis. In addition, polysomy 17 were noted in 43 cases (9.95%), of which 22 were co-amplified for HER-2/*neu* (5.09%). There were no significant associations of the polysomy 17 cases with any variable although the occurrence appeared to be almost three times higher in the older age group.

IHC was done to ascertain the expression of HER-2 proteins in tumour samples. Approximately a third of the samples (143/432; 33.10%) were positive (3+ reactivity) whereas 47 samples (10.88%) were negative (0, 1+ reactivity). In addition, a substantial number of cases (242/432; 56.01%) showed equivocal 2+ reactivity.

As per ASCO/CAP guidelines, we considered FISH as the gold standard for the diagnosis of HER-2/*neu* gene amplification. The comparison of results from the two assays is listed in [Table/Fig-1]. With the exclusion of the equivocal IHC cases, the overall concordance between the two assays was 94.12%. The positive and negative proportions of agreement were 91.61% and 97.82% respectively. The kappa coefficient was 0.851(SE – 0.043; 95% CI – 0.767 to 0.936) which is indicative of a very strong agreement between the two tests per the scale proposed by Landis JR and Koch GG [20]. Contingency table analysis using the Fisher-exact test also showed a significant association between the two assays (p<0.00001). Furthermore, the IHC assay demonstrated a sensitivity of 99.24%, specificity of 81.82%, and positive predictive value of 92.91% and negative predictive value of 97.83%. Of the equivocal IHC cases, 68 (28.10%) were FISH amplified for HER-2/*neu*, 168 (69.42%) were not amplified and the rest were FISH equivocal. Polysomy 17 was observed in 23 (5.32%) of the IHC equivocal cases.

FISH HER-2/neu	HER-2 protein expression by IHC		
	Positive (3+)	Negative (0/1+)	Equivocal (2+)
Amplified	131	1	68
Non-amplified	10	45	168
Equivocal	2	1	6

[Table/Fig-1]: Comparison of FISH results with IHC (n=432).
FISH – Fluorescent in situ hybridization, IHC – Immunohistochemistry

ER/PR receptor expression

Approximately, 2/3rd (284/432, 65.74%) of our cases were positive for ER receptor expression whereas 59.72% (258/432) of the cases were PR positive. Analysing the correlation between FISH HER-2/*neu* status and ER/PR expression demonstrated that ER and PR expression was higher in HER-2/*neu* negative tumours compared to HER-2/*neu* positive tumours. These inverse associations between the expression of ER/PR and HER-2/*neu* amplification were significant in contingency table analyses using the Fisher-exact test (p<0.00001). Interestingly, a substantial number of HER-2 positive tumours still expressed ER/ PR [Table/Fig-2].

		FISH HER-2/neu	
		Amplified	Non-amplified
Age	<50 years	82	81
	>50 years	118	142
ER status	Positive	107	171
	Negative	93	52
PR status	Positive	89	163
	Negative	111	60

[Table/Fig-2]: Correlation of FISH HER-2/neu status with age, ER/PR status.

DISCUSSION

Of the variety of methods available to determine the HER-2/*neu* status in breast cancers, FISH and/or IHC are the most viable for both clinical practice and research [21]. IHC measures the HER-2 protein expression whereas FISH measures copy number of the HER-2/*neu* gene. Given this scenario, one might expect some disagreement between the two tests. This study was undertaken to observe the concordance between IHC and FISH in evaluating HER-2/*neu* status at a tertiary cancer referral laboratory.

In our study, 46.29% cases demonstrated HER-2/*neu* gene amplification by FISH. This is higher than the range (18-30%) reported in various studies [22,23]. This may be a possible referral bias as our laboratory is a tertiary cancer centre. Indeed, higher rates of HER-2/*neu* amplification have been reported from similar centers [24,25]. An equivocal FISH result was observed in nine cases (2.08%). This is within the less than 3% range recommended by the ASCO/CAP guidelines [16].

As with other studies [26], the proportion of patients less than 50 years having HER-2/*neu* gene amplification was higher in our series (82/170; 48.23%). Surprisingly, this trend was repeated with ER positivity as well, which contrasts with other studies where the proportion of ER positive younger patients is lower [27,28]. This discrepancy may again be due to a selection bias in referral patients or due to the diversity inherent in our referral population. The overall concordance between FISH and IHC in our case series was 94.12%. This is similar to the results of other studies [16,21,29]. The kappa coefficient also demonstrated a very good agreement between the two tests, corresponding to near perfect agreement. Both the positive and negative concordances satisfied the ASCO/CAP guidelines. The IHC false positive rate of 7.09% for HER-2/*neu* was in the range seen in earlier studies [19,30]. Also, studies have shown that 3%-15% of invasive breast cancers over-express HER-2 protein without gene amplification [31]. Although false positives are considered to be the most common problem with IHC based HER-2/*neu* testing, there is little detail regarding their specific causes [32,33]. Putative reasons include increased receptor expression without genetic alteration caused by transcriptional or post-translational activation, artifactual high sensitivity of IHC assays and gene amplification below the level of detection of FISH assays. In contrast, there was a single false negative case. Reasons for this could be insufficient tissue preservation causing poor protein detection, scanty levels of gene amplification, down regulation of transcriptional and post-transcriptional/post-translational events leading to poor HER-2 protein levels or aberrant epitope production [26,34,35].

Our study had a substantial number of IHC equivocal cases reflecting the wide range of protein expression profiles which make them qualify for equivocal interpretation. Of these, 28% had gene amplification. This observation concurs with the report of a 23.9% incidence of IHC equivocal cases found amplified for the HER-2/*neu* gene by FISH [16]. IHC equivocal cases which did not show amplification (69.42%) are likely to be due to variation in pre-analytical factors like tissue fixation and processing which are inherent to referral samples affecting the epitope retrieval process [26,36,37].

The identification of true polysomies presents significant challenges in FISH assays with the incidence of polysomy 17 ranging from 10–50% [38,39]. In our study, polysomy 17 was seen in 43 cases, out of which 41.86% were IHC positive and 53.49% were IHC equivocal cases. This has been observed in other similar studies leading us and other authors to consider polysomy 17 as one of the major causes of equivocal results [39-42]. A point of interest is that, apart from HER-2/*neu*, chromosome 17 contains several other genes implicated in tumourigenesis like BRCA1, TOP2A and TP53. This raises the possibility that polysomy 17 might influence clinicopathologic and prognostic variables due to the altered expression of these growth-regulatory genes/proteins. Indeed, several studies have linked elevated CEP17 count ('polysomy') with unfavourable pathologic features as compared to disomic tumours [39,43]. Also, we found polysomy 17 and HER-2/*neu* amplification rate of 5.09% which is consistent with its rarity as reported in a large series of breast cancer [44].

A significant inverse relationship was noted between ER/PR status and HER-2/*neu* gene amplification. Similar results have been reported elsewhere and attributed to intricate receptor cross-talk between growth signalling pathways [24,45]. Despite this inverse association, our study revealed that 25.30% of cases were positive for both ER and HER-2/*neu*. This is important as co-positivity is imputed to decrease the efficiency of selective ER modulators like tamoxifen by facilitating cross talk between ER and HER-2/*neu* leading to membrane initiated steroid signalling [46]. This increases the oestrogen agonistic activity of tamoxifen leading to enhanced tumour growth and a possible reason for de novo resistance to tamoxifen [47]. Further, these types of tumours have been shown to possess more aggressive characteristics [48].

LIMITATION

One of the limitations of this study is that the findings may not be generalized to the entire Indian population as the samples come from a tertiary referral laboratory. Another is that pre-analytical variables like fixation time and tissue processing could not be controlled for referral FFPE samples.

CONCLUSION

In conclusion, our study demonstrated a highly significant concordance between FISH and IHC assays. However, inevitable numbers of equivocal cases in IHC, due to its subjective grading system, renders it ineffective as a gold standard test for HER-2/*neu* status in invasive breast cancers. FISH should always be used to determine true gene amplification when the IHC results are equivocal and false positive to exclude the possibility of polysomy associated protein expression and thus, permit an accurate choice of therapy.

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