INTRODUCTION
Breast cancer is one of the most common malignancies in the world and comprises of 22.9% invasive carcinoma and 16% of all cancers in females.\(^1\,\^2\) Since the first report of c-erb-b2 as a prognostic factor in 1987,\(^2\) researches have shown that about 20-30% of the breast cancer patients have Her-2/neu gene amplification, that is associated with a more aggressive phenotype and decreased survival. However, this underwent a volte-face with the advent of humanised anti Her-2/neu monoclonal antibody transtuzumab in Her-2/neu positive breast cancers, which is effective only if the detection of Her-2/neu status is accurate.\(^3\) In a small proportion of cases, protein overexpression may be found without gene amplification or gene amplification can be found in negative immunohistochemistry cases.\(^1\) Methods available to detect the Her-2/neu status are immunohistochemistry (IHC) and fluorescence In Situ hybridization (FISH), chromogenic In Situ hybridization (CISH) and polymerase chain reaction (PCR).\(^1\)

Evaluation of Her-2/neu status has become pivotal in determining patient’s eligibility for transtuzumab treatment.\(^4\) Although FISH analysis is especially suggested for scores of 2+, there are reports that recommend the FISH assay as a supplement to all scores of immunostaining. Because using FISH as a screening test is expensive and time consuming, many studies have advocated the combined approach of IHC as a primary screening modality followed by FISH assay for IHC inconclusive cases. In this situation, concordance between the results of IHC and FISH is of major importance.\(^5\)

IHC evaluation of Her-2/neu status is a simpler and more practical method that can be performed at a low cost in all pathology laboratories.\(^4\) However, IHC testing has its limitations. FISH is a highly accurate method with excellent sensitivity and specificity for the detection of Her-2/neu gene amplification and is considered to be the best indicator for beginning transtuzumab treatment in the breast cancer patients, which is costly and toxic.\(^5\) Since, this technique requires fluorescent microscope with technical expertise, both in performance and interpretation, it is performed in only specialised centres in Pakistan.

The primary aim in this study was to compare the results of Her-2/neu expression by IHC and gene amplification by FISH, as very limited local studies are available on Her-2/neu by FISH.

Abstract
Objective: To investigate the concordance and discordance between the test results of Her-2/neu by immunohistochemistry (IHC) and fluorescence In Situ hybridization (FISH) in breast cancer cases.
Study Design: Descriptive cross-sectional study.
Place and Duration of Study: Department of Histopathology, Dr. Ziauddin Hospital, Karachi, from 2011 to 2016.
Methodology: Forty-three specimens of invasive ductal carcinoma of breast were evaluated for grade and Her-2/neu status using IHC and FISH methods. Concordance and discordance between their results was determined.
Results: There is 100% concordance between FISH and IHC in cases scoring 0, 1+ (negative) and 3+ (positive) immunostaining. Tumour cases scoring 2+ immunostaining showed amplification in 69.2% cases. All grade-I tumours were non-amplified on FISH, while most of the grade-III tumours showed Her-2/neu amplification on FISH. There is significant association of Her-2/neu IHC with tumour grade and FISH (p<0.05). A fairly high proportion i.e. 69.7% of cases showed Her-2/neu gene amplification. There was high concordance between Her-2/neu testing on IHC and FISH, (Kappa co-efficient 0.466, p <0.001).
Conclusion: Her-2/neu amplification increases with increasing grade of breast cancer. A high proportion of Her-2/neu gene amplified cases indicates aggressive disease in that area and need for FISH testing on large scale, which is the gold standard for equivocal cases on immunohistochemistry.

Key Words: Her-2/neu invasive ductal carcinoma. Immunohistochemistry (IHC). Fluorescence In Situ hybridization (FISH).
METHODOLOGY

Cases of invasive breast carcinoma accessioned at Histopathology Department, Dr. Ziauddin Hospital, Karachi, from 2011 to 2016. All cases were fixed in 10% neutral buffered formalin and embedded in paraffin wax. For each case, 4 µm thick tissue sections cut from representative blocks and applied to positively charged slides. IHC testing was performed initially. Later on, focus of tumour showing IHC staining 0, 1+, 2+ & 3+ was subjected to FISH testing. Only those cases of invasive carcinoma breast in which Her-2/neu was evaluated by both IHC and FISH methods were included in the study. Specimens showing non-invasive carcinoma, malignancies other than carcinoma, and tumours in which FISH testing was not possible were excluded. Demographics of patients, tumour type, Her-2/neu results both by immunohistochemistry and Her-2/neu FISH were recorded. Standard ethics and maintenance of confidentiality of reports were assured. Due to high cost of Her-2/neu by FISH, it was performed only on a limited number of cases.

Immunohistochemical staining was carried out using an anti Her-2/neu (SP3) antibody. In carcinoma breast, Her-2/neu on immunohistochemical stain was considered positive (3+) when >10% of invasive tumour cells showed complete, intense circumferential staining.6

After deparaffinization and quenching, antigen retrieval was performed in citrate. Antibody titre was 1:200. Then secondary antibody was applied followed by chromogen DAB, counter-stain and mounting. Cases of carcinoma breast showing Her-2/neu 3+ by IHC was taken as positive control, parallel to the patient sample on same slide.

FISH analysis was performed using the Path Vysion Her-2/neu probe kit according to the manufacturer's guidelines and instructions. The kit uses two fluorescent labelled probes. LSI (locus-specific identifier) Her-2/neu is specific for the Her-2/neu gene locus (17q11) and CEP (centromere enumerator probe) 17 is specific for the alpha satellite DNA sequence at the centromeric region of chromosome 17. Manual micro-dissection was performed on cases to include only invasive carcinoma and to exclude ductal carcinoma In Situ and normal breast tissue. Briefly, slides were deparaffinized, pretreated and fixed in buffered formalin before performance of assay with the kit. The FISH procedure included denaturation of specimen DNA, probe preparation, hybridization, post-hybridization washes, and finally signal enumeration. Signals were counted for 20-50 tumour cells within an area of invasive carcinoma by two observers. The ploidy status of chromosome 17 was determined by calculating the average number of copies of CEP 17 based on a count of 60 cell nuclei. Chromosome 17 polysomy was defined as a CEP 17 of 3 or more. Her-2/neu gene amplification and positive result from FISH was defined as a HER-2/NEU:CEP 17 ratio >2 or equal to 2 (Figure 1).4,5

For data analysis, SPSS-16 was used. The results were given in the text as frequency with percentage for qualitative/categorical variables to compare percentage of qualitative/categorical variables in breast cancer cases using Chi-square test. Age was given in mean ±SD. Kappa coefficient for concordance was determined. P-value <0.01 was considered significant.

RESULTS

A total of 43 cases of invasive ductal carcinoma of breast between the ages of 25-76 years (mean 47 ±12 years) were included in this study. Status of grades of breast carcinoma cases, Her-2/neu by IHC and FISH are summarised in Table I.

Out of 43 cases of breast carcinoma, invasive ductal carcinoma NOS type comprised 41 (95%) of cases, in which one (2%) case was of grade-I carcinoma, which scored IHC 1+ and was FISH non-amplified.

Out of 15 (35%) cases of grade-II carcinoma, invasive ductal carcinoma NOS type comprised 41 (95%) of cases, in which one (2%) case was of grade-I carcinoma, which scored IHC 1+ and was FISH non-amplified.

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Polysomy-17 was noted in a single case (6.25%) of grade III carcinoma.

Figure 1: Carcinoma breast specimen evaluation: (a) Carcinoma Breast (H&E x 40), (b) Her-2/neu IHC 3+ Staining, (c) Her-2/neu FISH Amplified.
3+ staining on IHC was seen in 4 (19%) grade III tumour and all were FISH amplified. Grade was not available due to scanty tumour in 4 (9%) cases. However, these were included in study as both tests were performed. Among these, one (25%) case showed no immuno-staining on Her-2/neu and was FISH non-amplified, two (50%) cases scored 2+ staining on immunohistochemistry, in which one (50%) case was FISH amplified while other was FISH non-amplified, 3+ staining on immunohistochemistry was seen in a single case (25%) and was FISH amplified. One (2%) case was grade-III metaplastics squamous cell carcinoma, showing IHC 2+ staining and was FISH amplified. Other case was grade-I, tubular carcinoma showing no staining on immunohistochemistry and was FISH non-amplified (Table I).

There was significant association between immunohistochemistry and tumour grade with fluorescence In Situ hybridization (p<0.05, Table II).

Cases of invasive ductal carcinoma breast scoring 0 and 1+ (Negative) staining on IHC were five and all FISH non-amplified, so concordance rate was 100%. 2+ immunostaining was scored by 26 cases. Among these, 18 (69.2%) cases were Her-2/neu amplified, eight (30.8) cases were Her-2/neu FISH non-amplified so concordance was 69.2% and discordance was 30.8%. Twelve cases of carcinoma breast showed 3+ immune staining and all were Her-2/neu FISH amplified, showing 100% concordance (Table II). Kappa coefficient is 0.466 and p value <0.001. Most of cases, i.e. 30/43 (69.7%), were Her-2/neu FISH amplified while 13/43 (30%) were non-amplified. Association between the tumor grade, IHC and FISH is given in Table III.

**DISCUSSION**

Present study compared IHC and FISH methods using FDA approved kits. Her-2/neu overexpression and amplification increased with increasing grades of malignancy in the breast cancer specimens.

A report from Turkey shows that all nine cases of grade carcinoma breast were negative on immune staining as well as Her-2/neu FISH non-amplified which is nearly identical to the present findings. A local study also reported Her-2/neu FISH amplification in 0.8% of grade-I tumours, while 4% cases were non-amplified. A study from USA showed negative immune staining in 21% of grade-I tumours, out of which 89% were Her-2/neu FISH non-amplified and 11% were Her-2/neu FISH amplified. They also reported amplification in 9% cases of IHC negative grade-II tumours while 91% were non-amplified. In IHC 2+ grade-II tumours, 14% showed Her-2/neu FISH amplification and 86% were non-amplified.
In IHC 3+ grade-II tumours, 62% were Her-2/neu amplified and 38% were Her-2/neu non-amplified. These findings are pretty near to the present results.

A study from Egypt showed Her-2/neu amplification in 13.2% cases of grade-II carcinoma breast, while 86.8% cases were Her-2/neu FISH non-amplified. A local study reported Her-2/neu amplification in 41.4% grade-II tumours while 43.1% cases were non-amplified. These findings are near to our observations.

A study from Serbia showed positive relation between protein overexpression and high histological grade similar to the present findings. An American study showed negative immune staining in 30% grade-III tumours, out of which, 6% were Her-2/neu FISH amplified and 94% cases were Her-2/neu FISH non-amplified. They also reported 2+ immunostaining in 24% grade-III carcinoma, among which 27% were Her-2/neu FISH amplified and 73% cases were Her-2/neu FISH non-amplified. Forty-six percent grade-III breast carcinoma scored 3+ staining, among which 73% were Her-2/neu FISH amplified and 27% Her-2/neu FISH non-amplified. A study from Egypt reported Her-2/neu FISH amplification in 33% cases of grade-III carcinoma and non-amplification in 66% cases. A local study reported Her-2/neu FISH amplification in 24% and non-amplified in 13% cases of grade-III carcinoma breast.

These observations are not far from the present findings of greater frequency of amplification in higher grade tumours. Although Her-2/neu amplified tumors were mostly grade-III, none of the cases with Her-2/neu gene amplification presented as grade-I tumor in current study.

Studies from Turkey, Taiwan, India and Iran showed 100% concordance between Her-2/neu negative IHC and FISH similar to the present results. Studies from Switzerland and USA reported 99%, 95% concordance and 1%, 2% discordance between Her-2/neu FISH and Her-2/neu IHC in cases showing negative immunohistochimistry. Other studies from Turkey, India, and Saudi Arabia reported concordance of 80%, 85%, 87% and 84% in cases with negative immunostaining and Her-2/neu FISH, respectively. These frequencies are slightly lower than the present findings, which may be possibly due to sample size difference. Studies from India reported gene amplification in 70% and 66.6% cases and non-amplification in 30% and 30.5% cases, respectively, which is near to these results. Studies from Taiwan and India showed 53.3% and 25%, Her-2/neu amplification on FISH and non-amplification in 46.7% and 73% cases, respectively. Studies from Switzerland, America, Spain and Iran reported gene amplification in 95%, 7.1%, 18%, and 32.4% cases while gene was not amplified in 5%, 88%, 81%, and 67.6% cases, respectively. Studies from Turkey, Iran, and Saudi Arabia showed 23%, 27.9% and 23% and gene amplification in cases scoring 2+ immunostaining.

Concordance between IHC scoring 3+ and Her-2/neu gene in Indian studies, was 93.9% and 92.9% and discordance of 3.65% and 7.1% between IHC scoring 3+ and Her-2/neu FISH, respectively. Studies from Iran, Taiwan, and Switzerland reported concordance of 81.2%, 83% and 84% and discordance of 18.7%, 16.7% and 16% in cases with IHC staining 3+ and Her-2/neu FISH, respectively. Studies from India showed concordance of 78% and 85% and discordance of 21.8% and 14.2%, respectively which is in stark contrast to current study. A local study and studies from America, Iran, and Saudi Arabia reported concordance rate of 74%, 69%, 61.5% and 50%, respectively. A Turkish study reported amplification in 75% in IHC 3+ cases which is contrary to the present results.

Most of the studies including the present one showed an overall high concordance between IHC3+/FISH amplified and IHC negative/FISH non-amplified groups. The discordant results between IHC and FISH have been mostly attributed to 2+ scores on IHC, indicating that Her-2/neu+ connotes uncertainty and is an indicator of undetermined Her-2/neu status. The main problem appears to be underscoring of Her-2/neu on IHC.

Although there is good correlation between Her-2/neu gene amplification and protein overexpression, many studies also have shown that 3-15% of breast carcinoma overexpress the Her-2/neu protein without gene amplification and a small subset of breast carcinoma amplify the Her-2/neu gene without overexpression. Factors that may cause the discordance between the two techniques are usually related to IHC.

The most common reason identified for immunohistochemically positive, FISH negative discordance is interpretation of the immunohistochemical staining characteristics. Common issues with interpretation were interpreting weak to moderate circumferential staining as intense staining, interpretation in areas of crush and interpreting granular staining as uniform membranous staining or antibody clone used.

It is known that IHC can give different results due to subjective interpretation. Discordance among the results was attributed to the evaluation process of the samples, staining procedures and both evaluation and staining procedures. Some of the reasons for disparity between IHC 2+ and FISH results included presence of polysomy, intratumoral heterogeneity, variation in fixation, tissue processing, and tumour selection. It could also be due to the different antibodies available in the market for IHC Her-2/neu evaluation. Therefore, nationwide multicentric studies with a standard protocol should be performed for analysis to arrive at a generalised conclusion. A plausible number of cases, i.e. 30/43 (69.7%) show Her-2/neu amplification. This observation is closer to 74% and 91%. In some studies, proportion of Her-2/neu amplified cases was reported as 28.8%, 46.29% and 44.9%, Reports of Her-2/neu gene amplification 74% and 91% which is similar to these findings.
CONCLUSION

The present study showed a significantly high proportion of Her-2/neu gene amplification in breast cancers indicating aggressive disease in this area. The present study also supported the hypothesis that the consistency between IHC and FISH is higher for samples with 0,1+ and 3+ but variable for samples with 2+ and FISH remains a gold standard. Due to lack of substantial local data on Her-2/neu testing by FISH in Pakistan, studies on larger scale are recommended, specially in high grade tumours.

REFERENCES


