Abstract. Background: Amplification of the HER-2/neu oncogene and concomitant over-expression of its protein are detected in approximately 18% of invasive ductal carcinoma of the breast and is associated with poor prognosis. This study tested the use of fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC) to evaluate the HER-2/neu gene status and to ascertain the concordance rate between the two methods. Patients and Methods: Eighty two tumour samples containing representative tumour were divided for testing using each assay. HER-2/neu gene amplification is scored as a ratio of HER-2/neu gene amplification to chromosome 17. The ratio should be >2.2 to be considered as positive. 20 cells should be counted and an average score taken. An extra 20 cells should be counted if the ratio is between 1.8-2.2. Results: Seventy five effective samples were used. HER-2/neu gene was amplified in 19 out of 75 cases (25%) whereas, HER-2 protein, by IHC was over-expressed in 18 out of 75 cases (24%). In the 44 negative cases by IHC analysis only 7 cases (16%) of them showed amplification by FISH. Three out of 13 cases (23 %) scored as +2 showed gene amplification by FISH while 9 cases out of 18 cases (50%) were scored as +3. High concordance with FISH results 37:44 (84 %) was noted in negative cases (0/+1 cases), while lower concordance 3:13 (23 %) was seen in +2 cases. Conclusion: This study revealed a significant concordance between FISH results and IHC results. The study also showed that HER2/neu amplification is higher in Saudi patients than other western populations. However, due to the inherent failures of the IHC assay, FISH should always be used when the IHC results are inconclusive. The rational algorithm for HER-2/neu testing would be to perform IHC first, followed by FISH to validate equivocal IHC results.

The heterogeneous disease group of breast cancer and its molecular subtypes has been characterised by many recurrent genetic abnormalities such as unbalanced chromosomal rearrangements, deletions and gene amplifications (1-3). A cursory literature review readily shows that the treatment of these cancers is assuming a special status befitting to the biological characteristics of the tumour rather than its clinico-pathological features (4-6). Therefore, gene amplification and protein expression are in sharp focus, as never before, for a genuine diagnosis of breast cancer, and in particular the search for HER-2 assay directed at breast cancer pathology (7).

The accuracy in HER-2/neu results is critical for the use of targeted therapies (8-10). In validation experiments, a close association between the HER-2/neu gene amplification and protein expression has been documented earlier by Western and Northern blot analysis (11). However, in contrast, in immunohistochemistry (IHC), deparaffinised - formalin - fixed tissue can be quite variable in its ability to identify HER-2/neu amplified tumours. A serious disparity also exists between HER-2 protein expression evaluated by IHC and fluorescence in situ hybridization (FISH) (12, 13). Conflicting trends have continued, even in recent studies, despite efforts to narrow the discrepancy between the two methods (14). For tumours that are moderately positive by IHC, further evaluation by FISH, staining 2+ by IHC instituting therapy is recommended (15).

One of the reasons for the discrepancy between the use of IHC analysis and FISH may arise from the use of formalin fixation, causing destruction of the HER-2 epitope thus
leading to a false-negative IHC result in approximately 15%-20% of HER-2/neu-positive cases. FISH may assist in overcoming certain misclassification errors that can occur as a result of the tissue fixation procedure used in IHC. A significant decrease in false-positive (IHC 3+/FISH−) results has been indicated through the introduction of an FDA-approved scoring system for HER-2/neu IHC using a normalised IHC score in breast cancer (16). Even when used alone, FISH is advantageous in that it is able to circumvent antigenic changes that occur in formalin-fixed, paraffin-embedded tissue; a major limitation of the IHC protocol. Unlike IHC, the use of FISH is limited by its relative expense, although it may be the overall more cost-effective option, since it is sensitive and highly specific and has the added advantage of being associated with most objective scoring system (17).

It is evident that there is an urgent need to re-evaluate the IHC scoring method, IHC being still the widely used assay in most diagnostic centres. Also, immediate efforts must be made to narrow the gap between IHC and FISH assessment. Such studies are rare in the Saudi Arabia, despite initial reports indicating the serious rise in breast cancer cases (20). The aim of this study was to evaluate the HER-2/neu gene status in Saudi patients and furthermore to ascertain the concordance rate between FISH and IHC techniques.

Patients and Methods

Patient population and tissue specimens. Breast cancer tissue samples from 82 randomly selected patients, aged between 24-81 years reporting for breast cancer complications at King Abdul Aziz University Hospital, Jeddah, during 2002-2008 were used for the study, although due to technical reasons, only samples from 75 patients were finally included. All samples were carefully avoided to minimise any damage to DNA leading to failure in FISH assay.

HER-2/neu FISH on paraffin section

Tissue de-paraffinisation. Slides were immersed in xylene for 10 min at room temperature; this step was repeated twice using fresh xylene each time, followed by dehydrating in 100% ethanol with two immersions of 5 min each; the slides were later air dried. These were subsequently immersed in 0.2 M HCl for 20 min and transferred to purified water and washed in buffer (2 × SSC, pH 7) for 3 min. Thereafter the samples were immersed in pretreatment solution at 80°C for 30 min. A volume of 50 ml of this solution was prewarmed at 80±1°C in a water bath, after which the slides were immersed in purified water for 1 min followed by immersion in wash buffer for 5 min, twice, to complete the washing. Excess buffer was removed by blotting edges of the slide on a paper towel and then the slides were immersed in protease solution at 37°C for 15 to 20 min; the pH of the protease buffer was kept between 0.8 - 1.5. The protease solution was prepared by adding 250 mg protease freshly before slide immersion to protease buffer, at 37±1°C. Slides were immersed twice in wash buffer for 5 min and allowed to air dry.

The slides were evaluated for the ability to assess appropriate protein digestion for some cases before moving on to hybridisation. Variable time intervals were enforced as some tissues were resistant to digestion. After incubating the slides in protease for 15 to 20 min, the slides were rinsed in 2 × SSC buffer for 10-20 s, then 10 μl of DAPI counter stain was added to the slides and was replaced the coverslip. The slides were viewed using DAPI filter (Illinois, USA) and the tissue section assessed for under-, appropriate- and over-digestion. Cells that were over-digested appeared ghostly and showed loss of cell border, and for them, digestion was repeated with new slides at a reduced time; those that were under-digested were gently wiped off the immersion oil and the cover slip was removed by soaking the slide in 2 × SSC buffer. The slides were transferred to fresh 2 × SSC buffer for some time to clean off any residual oil. Should further digestion be required, protease treatment was repeated, readjusting the time of treatment.

The prepared slides were treated with neutral buffered formalin (4% formaldehyde in PBS) at room temperature for 10 min followed by two washing steps in wash buffer of 5 min each and air dried before processing for hybridisation. All the steps were carried out under continuous shaking and at room temperature unless otherwise stated.

FISH protocol. The pH of the denaturation solution, formamide being critical, was maintained at pH 7.0-7.5 at room temperature and for use, transferred to an appropriate container kept in a water bath at 73±1°C. DNA was denatured by immersing the prepared slides for 5 min in this solution, after checking that the temperature was at 73±1°C, and then transferred to 70% ethanol for 1 min, agitated to remove excess formamide, dehydrated in graded ethanol, 70% to 85% and 100%, for a 1 min dipping in each change. Excess ethanol was drained off and the slides wiped and air dried ready to be used for probe labelling.

A PathVysion (Abbott Park, Illinois, USA) HER-2 DNA probe kit was used. The probe is composite, being composed of two probes; one LSI HER-2/neu DNA probe, with a 190 kb Spectrum Orange labelled with fluorescent DNA probe specific for the HER-2/neu gene locus (17q11.2-q12) and the other, a CEP 17 DNA probe; a 5.4 kb Spectrum Green labelled fluorescent DNA probe specific for the alpha satellite DNA sequence at the centromeric region of chromosome 17 (17p11.1-q11.1). Both probes were pre-mixed and pre-denatured in hybridisation buffer, needing just a mix and spin before use. The areas to be hybridised were marked with a diamond tipped scribe. A volume of 10 μl of probe mixture was added to the target area of the slide. A 22 mm × 22 mm glass coverslip was placed immediately over the probe and the probe mixture was allowed to spread evenly under the coverslip. Air bubbles were avoided since they interfere with hybridisation. The cover slips were sealed with rubber cement and placed in the pre-warmed HYBrite...
Fluorescence microscopy and interpretation of FISH results. The slides were subjected to fluorescence microscopy after gradually reaching room temperature. A total of 20 nuclei were enumerated and analysed according to the method described by Press et al. (21). The signals were carefully examined using Axioskop (Jena, Germany) and Axiosplan fluorescence microscopes (Jena, Germany) equipped with 100 watt mercury lamps, and narrow band pass filters (Vysis, USA). Images were captured by analogue camera. The depth of focus was adjusted and scanned at 25 × objective to view the hybridised area and the relocated target of interest was viewed at 100 × objective. Analysis commenced in the upper left quadrant of the selected area and scanned from left to right. The numbers of signals were counted within the nuclear boundary of each evaluable inter-phase cell. Areas of necrosis and nuclear border ambiguities were excluded from the count. Similarly, nuclei with signals requiring subjective judgment or having weak intensity and non-specificity and with noisy background or having insufficient counter stain were excluded. Nuclei with no signals or with signals of only one colour were also excluded; only those nuclei showing discrete signals were enumerated. Focus was given to those nuclei with one or more FISH signals of each colour. Split signals were counted as a single hybridization signal. The numbers of LSI Her2/neu and CEP 17 signals per nucleus were recorded in columns. Results on counting of 20 inter-phase nuclei from tumour cells per target were reported as the ratio of the total Her2/neu signals to those of CEP 17. Specimens with amplification showed a LSI Her2/neu: CEP 17 signals ratio ≥2.2, whereas normal specimens showed a signal ratio of ±1.8. Results at or near the cut-off point (1.8-2.2) should be interpreted with caution. In the event of a borderline result (1.8-2.2) should be interpreted with caution. In the event of a borderline result (1.8-2.2), particularly if there also appears to be variability of the counts from one nucleus to the other, 20 additional nuclei would be enumerated. The specimen slide was also re-enumerated by another technician to verify the results.
precise measure of HER-2 expression than IHC assays in formalin-fixed biopsies. Table III shows a comparative study of IHC and FISH in breast carcinomas for hypodysomy and polysomy 17, representing <2 and ≥2.25 copies per cell.

Non amplified and amplified signals of HER-2/neu gene with polysomy 17 during various observations are shown in Figure 1. Figure 2 shows the high signals monosomy 17 in non-amplified HER-2/neu and polysomy 17 without gene amplification.

The study further revealed that polysomy, hypodisomy and gene amplification can be detected accurately in individual cells by using FISH. Thus, a net increase in HER-2/neu gene copy number consecutive to polysomy 17 in the absence of specific gene amplification lead to a strong protein overexpression in a small subset of breast carcinoma observed in the present study.

Discussion

Incidence of breast cancer among Saudi women is very high, prompting this study which investigated the role of genetic abnormality in such cases of Saudi breast cancer patients. Prior studies (23) have reported that HER-2/neu gene amplification is more common in younger patients, and the present study highlighted a significant relationship between HER-2/neu gene amplification and young patient age.

Considerable molecular differences from various ethnic groups are frequently reported. In one such study performed to identify the potential differences between breast cancer with HER-2/neu amplification between Swiss and Saudi women, a higher incidence of grade 3 cancer with complete absence of low grade cancer and higher incidence of HER-2/neu amplification has great implications for Saudi women (24). The variation is possibly related to a difference in genetic susceptibility and lifestyle of the two ethnic groups. In Saudi study, the results have showed that Saudi women also have high incidence of higher grade cancer and HER-2/neu amplification.

Indications are that HER-2/neu amplification significantly infiltrates ductal carcinoma more than lobular carcinomas and that higher grade tumours are more likely to demonstrate HER-2/neu amplification than lower grade carcinomas (25). That study also demonstrated that all cases with HER-2/neu amplification were infiltrating ductal carcinomas and they were of high grade.

Importantly, the current study showed that the prevalence of HER-2/neu amplification is slightly higher in Saudi patients as compared to other published studies (26). This prevalence rate was almost similar to a Qatari study (23), which revealed that the provenance rate of HER-2/neu gene is over-expressed in 26% of Qatari breast cancer patients. However, the small sample size of Qatari study might not accurately reflect the true prevalence.

The use of the FISH technique not only helps to assess the HER-2/neu gene status but further permits the determination of the number of copies of the gene and their spatial distribution within the nuclei (27) and also to define the exact level of HER-2/neu gene amplification by dual colour FISH analysis (28). As such, FISH has gained considerable interest as a reliable and valid method for determining HER-2/neu status, and its prognostic utility has been discussed by many authors (29). Unlike other gene-based assays, Southern blotting or polymerase chain reaction, FISH is not hampered by dilution artefacts. Notwithstanding the substantial cost of FISH, it is being considered as gold standard for assessing HER-2/neu status (30).

There is however the potential to obtain more reliable results by adjusting incubation times and checking protease treatment at the appropriate stage, as demonstrated for the first time in the present study. Although cases with high levels of gene amplification consistently show a gain of chromosome 17, between three and five copies per nucleus (31), this is in contrast to the low and intermediate levels of HER-2/neu gene amplification, showing lower gains of chromosome 17 and DNA aneuploidy. However, a small proportion of the cases also reveal unamplified HER-2/neu gene with monosomy 17, as described elsewhere (32).

The low frequency of the inverse association of monosomy 17 and HER-2/neu gene amplification are both of great interest (32). Concordance rates as high as 80-90% have been reported in the detection of protein overexpression in immunohistochemistry with gene amplification (FISH) (16). In spite of the good correlation between HER-2/neu gene amplification and protein overexpression, 3% to 15% of breast carcinomas overexpress the HER-2/neu protein without gene amplification and a small subset of breast carcinoma amplify the HER-2/neu gene without overexpression (29, 33). In the present study, HER-2/neu gene amplification was consistently found to be associated with protein overexpression, except in cases scored as

Table III. Incidence of hypodisomy 17 (chromosome 17<2 copies per cell) and polysomy 17(chromosome 17>3 copies per cell) in comparison with IHC and FISH in breast carcinoma.

<table>
<thead>
<tr>
<th>Chromosome 17</th>
<th>FISH Score</th>
<th>IHC Score</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>+1</td>
<td>+2</td>
</tr>
<tr>
<td>Hypodisomy</td>
<td>Non amplified</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Amplified</td>
<td>-</td>
</tr>
<tr>
<td>Total cases of aneusomy 17</td>
<td>27</td>
<td>5</td>
</tr>
<tr>
<td>Polysomy</td>
<td>Non amplified</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Amplified</td>
<td>4</td>
</tr>
<tr>
<td>Total cases with polysomy 17</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>
Figure 1. Various levels of amplifications of HER-2 gene: (a) non amplified, where two green signals represented normal level of chromosome 17 and red signals represented normal level of HER-2 gene, (b) low level amplification without polysomy 17 and, (c) and (d): intermediate amplification in HER-2 gene with polysomy 17.

Figure 2. Strongly amplified signals (a, b); and the corresponding monosomy 17 in non-amplified HER-2 (c) and polysomy 17 without gene amplification (d).
overexpression of HER-2/neu should be investigated. FISH negative cases, especially in relationship to response and prognosis of +2, FISH positive cases and +3, additional clinical studies that correlate trastuzumab potential cardiac toxic effects of trastuzumab. Besides agreeing with Slamon et al. (36), the current authors tend to agree with Slamon et al. (36). The study also showed that HER2/neu amplification is high in Saudi patients and therefore these results should be considered when subjecting patients whose tumours show weak overexpression of HER-2/neu by the HercepTest to the potential cardiac toxic effects of trastuzumab. Besides additional clinical studies that correlate trastuzumab response and prognosis of +2, FISH positive cases and +3, FISH negative cases, especially in relationship to polysomy 17, should be investigated.

Conclusion

Considering the overall logistical difficulties, as well as the accuracy, time and cost for the double testing of HER-2/neu (IHC/FISH), gene analysis may be an efficient and useful approach for HER-2/neu screening of breast cancer patients, particularly for laboratories running a large number of breast cancer surgical specimens, where the pathological experience of the staff would guarantee a correct tumour grading. Importantly, this study showed that HER-2/neu amplification is slightly higher in Saudi patients as compared to other ethnic groups (26). There was a significant concordance between FISH results and IHC results. However, due to the inherent failures of the IHC assay, FISH should always be used when the IHC results are inconclusive. The rational algorithm for HER-2/neu testing would be to perform IHC first, followed by FISH to validate equivocal IHC results.

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Disclosure/Conflict of Interest

The authors have no conflicts of interest to declare. No part of this report has been presented elsewhere.

References


