Assessment of Her-2/neu gene amplification status in breast carcinoma with equivocal 2+ Her-2/neu immunostaining

Naglaa A.E. Mostafa a,*, Saad S. Eissa a, Dalia M. Belal b, Soheir H. Shoman a

a Pathology Department, National Cancer Institute, Cairo University, Egypt
b Biostatistics Department, National Cancer Institute, Cairo University, Egypt

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Abstract  Background and purpose: Amplification of Her-2/neu gene occurs in 25–30% of breast carcinomas. FDA approved trastuzumab (Herceptin) is effective only in tumors having the gene amplification. Immunohistochemistry (IHC) for Her-2/neu protein is widely used but false positive and false negative results exist. Fluorescence in-situ hybridization (FISH) has both excellent sensitivity and specificity in detecting Her-2/neu amplification. Comparative studies have shown discordant results in proportion of cases with equivocal 2+ immunostain. This study is thus conducted to ascertain the frequency of Her-2/neu gene amplification by FISH in breast carcinoma specified as score 2+ by IHC and to correlate these findings with parameters of prognosis in breast cancer.

Methods: From October 2008 till May 2010 all paraffin blocks from cases with invasive breast carcinoma which were scored as 2+ by IHC were included in the study. There were 50 cases. Immunohistochemical evaluation of Her-2/neu was performed using the HercepTest. All cases were immunohistochemically evaluated for ER and PR. FISH was performed using FDA approved Path-Vysion Her-2/neu/CEP 17 dual color probe.

Results: Nine cases (18%) out of 50 cases scored as Her-2/neu 2+ by IHC showed true gene amplification with a median value of scoring ratio 4.28 ranging from 2.37 to 13.26. Another two cases
showed low level of amplification but when corrected for Her-2/neu/CEP ratio they did not show true amplification as they were associated with polysomy 17. With the exception of tumor size, neither patient’s age, histologic grade nor lymph node status were correlated with Her-2/neu gene amplification. Significant inverse correlation existed between Her-2/neu gene amplification and ER ($P = 0.01$), PR status ($P < 0.001$).

**Conclusion:** Even though FISH is a more complex and expensive procedure, it should be considered the method of choice for assessment of Her-2/neu gene status especially for equivocal cases by IHC that are not accompanied by true gene amplification in the majority of breast carcinoma cases.

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**Introduction**

Her-2/neu (erbB2) proto-oncogene encodes a type I tyrosine kinase receptor that is homologous to the epidermal growth factor receptor and is located on the long arm of chromosome 17. Her-2 induces cell division and stimulates factors facilitating cell motility [1,2].

With the exception of small fraction of cases, overexpression of the protein product of Her-2/neu gene is usually a direct consequence of gene amplification. Amplification of Her-2/neu gene occurs in 25–30% of breast carcinomas and results in over expression of gene product [3,4]. Gene amplification of Her-2/neu oncogene is associated with rapid tumor growth, increased risk of recurrence after surgery, poor response to conventional chemotherapy and it predicts worse prognosis with significantly decreased disease free survival and overall survival [5,6].

Testing to determine Her-2/neu status has come into focus since the approval of trastuzumab for the treatment of Her-2 positive breast cancer. FDA approved trastuzumab (Herceptin) binds to the extracellular domain of Her-2/neu and blocks cell proliferation of tumor cells that overexpress Her-2/neu. It also induces antibody dependant cellular toxicity against tumor cells [7]. Lapatinib, Her-1/Her-2 dual receptor inhibitor is also being introduced as an effective modality treatment for patients expressing gene amplification. Unfortunately, the treatment is expensive and carries serious adverse effects like cardiotoxicity with Herceptin. Both drugs being effective only in tumors having the gene amplification [3,8].

Accurate assessment of erbB2 status in individual tumor cells is mandatory before application of specific therapy strategies and has thus taken on great importance in routine work up of breast cancer, complementing the prognostic and predictive markers used [9–11].

Various techniques are available for assessment of Her-2/neu status in tumor cells. These include detection of gene amplification (Southern blot, PCR, fluorescent in situ hybridization; [FISH], messenger RNA [Northern blot, RT-PCR] or amount of protein [Western blot, ELISA, immunohistochemistry; IHC]) [5,12].

Currently the two most widely used technologies for assessment of Her-2/neu are IHC and FISH, both of which can be applied on formalin fixed paraffin embedded tissues. FISH has both excellent sensitivity and specificity in detecting Her-2 amplification but with high requisite of special instrumentaion and expertise to perform and interpret the assay [3,13].

IHC for Her-2/neu protein is widely used but false positive and false negative results are present. Comparative studies have shown high concordance rates between IHC analysis and FISH in cases with IHC scores 0, 1+ and 3+. However, discordant results are significant in proportion of cases with equivocal 2+ immunostain [9,14,15].

This study is thus conducted to ascertain the frequency of Her-2/neu gene amplification in breast carcinoma specified as score 2+ by IHC and to associate these findings with clinicopathologic parameters (age, tumor size, lymph node metastasis,), in addition to estrogen receptor (ER) and progesterone receptor (PR) states.

**Materials and methods**

From October 2008 till May 2010 all Paraffin blocks from cases with invasive breast carcinoma, which were scored as 2+ by IHC were eligible for the study (50 cases). The tumor specimens of blocks were previously fixed in neutral buffered formalin and embedded in paraffin. Blocks from patients with metastatic or recurrent breast cancer were eligible for FISH study if they were scored as 2+ by IHC. Patients with pure duct carcinoma in situ (DCIS) were excluded from the study. Clinical and demographic data including patient’s age, tumor size, and lymph node status were recorded for each case.

Immunohistochemical evaluation of Her-2/neu was performed using the HercepTest (Dako, Glostrup, Denmark) according to the manufacturer’s instructions. The assessment of Her-2/neu overexpression was performed as recommended by the following HercepTest scoring guidelines: 0; no staining or less than 10% membrane staining, 1+; partial membrane

![Figure 1](image-url) Immunohistochemistry score 2+ Her-2/neu in a case of breast carcinoma (200×).
staining in greater than 10% of the tumor cells, 2+; weak or moderate complete membrane staining in greater than 10% of the tumor cells (Fig. 1), 3+; strong complete membrane staining in greater than 10% of the tumor cells (HercepTest guidelines).

All cases were immunohistochemically evaluated for hormone receptor state (ER and PR) according to Allred score [16]. In brief, a total scoring of proportion scoring (PS) and intensity scoring (IS) was done with 0–2 considered negative and 3–8 considered positive.

Fluorescence in-situ hybridization (FISH) was performed using FDA approved Path-Vysion Her-2/neu DNA probe kit (Abbott, Molecular Inc., IL, USA) which contained two labeled DNA probes: the Her-2/neu probe which comprises locus specific identifier (LSI) Her-2/neu gene labeled in spectrum orange, and chromosome enumeration probe 17 (CEP 17) which is labeled in spectrum green and hybridizes to the alpha satellite DNA located at the centromere of chromosome 17 (17p11.1). Inclusion of CEP 17 relative to the copy number of chromosome 17 allows the copy number of the Her-2/neu gene to be determined.

Tissue sections prepared from corresponding paraffin blocks were mounted on poly-L-lysine coated glass slides. Slides were air dried and baked overnight at 56°C, dewaxed in xylene followed by immersion in 100% ethanol for 2 min. Pretreatment of the sections for 10 min at 70°C, followed by pepsin digestion at 37°C for 15 min was done. Before denaturation, slides were warmed at 60°C and an appropriate amount of hybridization mixture was applied onto the tissue sections according to the size. Co-denaturation and hybridization were simultaneously done using Tissue Hybite (Vysis).

Slides were then washed with 0.4% SSC wash buffer, and counterstained with DAPI. Scoring was done using Zeiss axi-oscope fluorescent microscope (Zeiss Ltd., Germany) equipped with specially designed excitation and emission filter sets, dual band and triple bandpass filters, and cooled CCD camera. For each case the following factors were noted: adequacy of digestion, intensity of fluorescent signals, nonspecific fluorescent background, tissue histology, and areas with non-overlapping nuclei. Thirty randomly selected invasive nuclei in each of the two separately distinct microscopic areas were evaluated (a total of 60 per case). Results on the enumeration of 60 interphase nuclei from tumor cells per target were reported as the ratio of the average Her-2/neu copy number (orange) relative to that of CEP 17 (green). Other features like polysomy 17 were noted.

The interpretation of the FISH assay was done following the ASCO/College of American Pathologists (CAP) guidelines. A ratio of HER-2/neu to CEP 17 signals higher than 2.2 was reported as amplification, a ratio less than 1.8 was reported as non-amplification, while a ratio between 1.8 and 2.2 was taken as an equivocal result. A recounting of additional 20 tumor nuclei (i.e. total 80) was done for the equivocal cases. The assay was repeated in case of failure due to technical problems like excess paraffin in the tissue, excess background autofluorescence, no gene signal or improper protease digestion. Normal and amplified control slides, Probe Check, were run simultaneously with the test cases. The slides were then stored in the dark at −20°C. Cases were scored independently by two observers.

Statistical evaluation

Statistical package for social science (SPSS) version 12 was used for data management and analysis. To test the difference between groups with respect to quantitative variables,

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Number</th>
<th>Her-2/neu by FISH</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Non-amplified (n = 41)</td>
<td>Amplified (n = 9)</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td>n %</td>
<td>n %</td>
</tr>
<tr>
<td>≤35</td>
<td>13</td>
<td>11 (84.6)</td>
<td>2 (13.4)</td>
</tr>
<tr>
<td>&gt;35</td>
<td>37</td>
<td>30 (81.1)</td>
<td>7 (28.9)</td>
</tr>
<tr>
<td>Median Range</td>
<td></td>
<td>43</td>
<td>43</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td></td>
<td>25-72</td>
<td>28-55</td>
</tr>
<tr>
<td>Mean, SD</td>
<td></td>
<td>3.2, 1.7</td>
<td>4.7, 2</td>
</tr>
<tr>
<td>Histologic grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GHII</td>
<td>38</td>
<td>33 (86.8)</td>
<td>5 (13.2)</td>
</tr>
<tr>
<td>GHIII</td>
<td>12</td>
<td>8 (66.7)</td>
<td>4 (33.3)</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>7</td>
<td>7 (100.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Positive</td>
<td>43</td>
<td>34 (79.0)</td>
<td>9 (21.0)</td>
</tr>
<tr>
<td>Number of LNs</td>
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</tr>
<tr>
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<td>2 (11.1)</td>
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<tr>
<td>&gt;3</td>
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<td>18 (72.0)</td>
<td>7 (28.0)</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>Positive</td>
<td>42</td>
<td>39</td>
<td>3</td>
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<tr>
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<td>12</td>
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<td>6 (50.0)</td>
</tr>
<tr>
<td>Positive</td>
<td>38</td>
<td>35 (92.1)</td>
<td>3 (7.9)</td>
</tr>
</tbody>
</table>

Table 1 Correlation of Her-2 status with clinical, histopathologic and ER, PR status.
Mann–Whitney test was used. To compare percentages, Chi square test and fisher exact were used. Spearman correlation coefficient was calculated to test the association between numeric and ordinal variables. Significant level was ≤0.05.

Results

Clinical and histopathologic parameters

All patients were females with mean age of 43.9 years and a standard deviation of ±11.3 years. The age range was from 28 to 72 years. Twenty-six patients were ≤35 years, while 74% were >35 years of age. Forty-eight cases (96%) were invasive duct carcinoma while 2 cases were invasive lobular carcinoma. As regards tumor size, 11 cases (22%) were T1 (<2 cm); 31 cases (62%) were T2 (2–5 cm); while 8 cases (16%) were T3 (>5 cm). Twelve cases (24%) were grade 3 while the remaining 76% were all grade 2. Four cases showed extensive intra-duct component in the form of comedo, solid and cribriform patterns while lymphovascular invasion was detected in three cases. Lymph node metastasis was present in 43 cases (86%), N0 cases represented 14% of the cases, N1 (1–3 LNs) was reported in 18 cases, N2 (4–10 LNs) 16 cases, while N3 (>10 LNs) was documented in 9 cases. Capsular nodal invasion was present in about 6 cases.

Immunohistochemical ER and PR evaluation

Eight cases were ER negative and 12 cases were PR negative. Seven cases showed both ER and PR negative cases. As regards the relation between ER and PR it was highly significant since 7 cases were both ER and PR negative and 37 cases were both ER and PR positive, P value < 0.001 (Table 1).

FISH interpretation

Out of 50 cases scored as Her-2/neu 2+ by IHC and investigated for FISH, 9 cases (18%) showed true gene amplification (Figs. 4 and 5) with a median value of Her-2/CEP 17 ratio of 4.28 ranging from 2.37 to 13.26.

Another two cases showed low level of amplification but when corrected for Her-2/neu/CEP ratio they did not show true amplification as they were associated with polysomy 17 (Fig. 3). Polysomy 17 thus accounts for about 4% of our cases. Thirty-nine cases showed no amplification (Fig. 2).

One case showed expression of ER, PR and Her-2/neu gene amplification (triple positive). The case was grade 3, tumor size >2 cm and with >10 positive nodes. Two cases showed neither expression of ER, PR nor Her-2/neu gene (triple negative).
FISH amplified cases [9,19]. ASCO/CAP guidelines reported showed amplification range from 6% to 25% of IHC 2+ about 18%. This is comparable to previous studies that score 2+ by IHC that showed true gene amplification was reported significant higher rates [8,15].

accompanied by true gene amplification [2,7,9], while others ies showed that score 2+ Her-2/neu by IHC is infrequently by FISH when IHC results are equivocal [17–19]. Several stud-

ment of cases of invasive breast cancer by IHC supplemented used. Nevertheless, many centers recommend the initial assess-

ment of amplified cases due to chromosome 17 polysomy. On 2+ Her-2/neu gene (p value 0.03) with a mean size of 4.7 cm among amplified cases compared to 3.2 cm among non-ampli-
ed cases. Two out of 18 cases (11.1%) with LN ≤ 3 showed amplified Her-2/neu compared to 7 cases (28%) out of 25 with LN > 3 showing amplified Her-2/neu (P = 0.138) (Table 1).

Seventy-five percent (75%) of ER negative cases were amplified compared to 7.1% among ER positive cases with P < 0.001. On the other hand 50% of PR negative cases were amplified compared to 7.9% among PR positive cases with a statistically significant difference, P = 0.001. No significant relation was found between tumor grade and Her-2/neu amplification (P = 0.113). There was good positive correlation between tumor size and lymph node positivity (r = 0.57, P < 0.001) (Table 1).

Discussion

Evaluation of Her-2/neu gene status has become part of the core dataset in pathologic reporting of invasive breast cancer [3,10]. Despite some evidence that FISH testing predicts the therapeutically significant Her-2/neu status more accurately, the approach of primary immunohistochemistry screening with supplementary FISH molecular confirmation is widely used. Nevertheless, many centers recommend the initial assessment of cases of invasive breast cancer by IHC supplemented by FISH when IHC results are equivocal [17–19]. Several studies showed that score 2+ Her-2/neu by IHC is infrequently accompanied by true gene amplification [2,7,9], while others reported significant higher rates [8,15].

In this study, the percentage of cases assigned as Her-2/neu score 2+ by IHC that showed true gene amplification was about 18%. This is comparable to previous studies that showed amplification range from 6% to 25% of IHC 2+/ FISH amplified cases [9,19]. ASCO/CAP guidelines reported an incidence of 23.9% [20]. Such cases with score 2+ by IHC and with negative gene amplification are considered false positive by IHC. Possible explanations for these results could be attributed to variation in time fixation, processing conditions that can lead to variation in the intensity of staining especially with the use of heat inactivation epitope retrieval that can cause false positive results [18,21,22]. Tumor heterogeneity is another possible explanation which is documented in many tumors with the presence of a clone that is biologically different from nearby clones [23,24].

One case showed expression of ER, PR and Her-2/neu (triple positive). Her-2/neu amplification is reported to be associated with resistance to tamoxifen therapy. It is postulated that in these cases tamoxifen functions as an estrogen agonist to stimulate growth in breast cancer cells, which express an increased level of Her-2 and ER co activation resulting in denovo resistance for tamoxifen [25,26]. Two cases in this study showed triple negative results (ER, PR, Her-2/neu). Triple negative cases, which probably represent a specific type of breast cancer known as basal type, are also known to be resistant to tamoxifen with rapidly progressive clinical course [27,28].

Two of the cases in this study showed polysomy 17. The frequency of polysomy 17 varies from 10% to 50%. ASCO reported that 8% of equivocal cases exhibit polysomy 17. Several studies have confirmed that protein overexpression may be secondary to polysomy 17 with resultant increase in the copies of Her-2/neu gene in the absence of gene amplification. These are almost considered Her-2/neu negative tumors rather than tumors with true Her-2/neu gene amplification [29].

As regards clinicopathologic correlation, no statistically significant association existed between Her-2/neu gene amplification and age, grade or lymph nodes metastasis. This was also reported by other investigators [5,7]. Tumor size showed significant association with Her-2/neu gene amplification in concordance with other studies [14]. Many investigators found significant association with ER, PR hormone status and is considered an important guide for therapy [4,6–8]. Similar findings were reported in our study.

Even though FISH is a more complex and expensive procedure, it should be considered the method of choice for assessment of Her-2/neu gene status especially for equivocal cases by IHC that are not accompanied by true gene amplification in the majority of breast carcinoma cases. False treatment with trastuzumab in the positive cases by IHC not only leads to loss of money of the patient or the government but also it means psychological burden to the patient who was waiting for improvement with the expensive drug, this is added to the exposure to side effects of the drug without any benefit. So, although FISH is expensive, it is more cost effective in cases of score 2+ immunostaining. This is because the price of trastuzumab is much higher than the cost of performing FISH, in addition to the psychological burden of false treatment and side effects of the drug. Failure to detect FISH amplified cases would have an adverse effect on survival of these patients. FISH also has the great advantage of evaluation of chromosome 17 status at the same setting for correction of amplified cases due to chromosome 17 polysomy. On the other hand, IHC expression without gene amplification appears to belong to a better prognostic group; failure to detect this group would have negative effect on survival of these patients.
References


