Evaluation of HER2 Gene Status in Breast Cancer by Chromogenic In Situ Hybridization: Comparison with Immunohistochemistry

SOPHIA KOUNELIS1, NIKIFOROS KAPRANOS2, NIKOLAOS MALAMOS1 and EFI KOURI-BAIRAKTARI1

1Departments of Pathology and Oncology, Elena Venizelou Hospital and 2Department of Pathology, Division of Molecular Pathology, MITERA Maternity and Surgical Center, Athens, Greece

Abstract. Background. The purpose of this study was to evaluate HER2 gene status in relation to chromosome 17 polysomy with the chromogenic in situ hybridization (CISH) technique and to compare the results with those of immunohistochemistry (IHC). Methods and Results. Sixty six cases of breast carcinoma with an immunohistochemical HER2 protein score of 1+, 2+ 3+ (HercepTest™) were investigated. HER2 gene status was evaluated on paraffin sections with the CISH technique using a digoxigenin-labeled DNA probe. In HER2 positive cases with low level amplification (LLA), the copy number of chromosome 17 was determined. Thirty four tumors (51.5%) were negative and 32 (48.5%) were positive for HER2 gene amplification. Of these 10 tumors (15%) showed LLA and 22 tumors (33.5%) high level amplification (HLA). Nine of ten tumors with LLA had an equal or greater than two ratio of HER2 to chromosome 17 signals. The correlation of the results obtained by CISH and IHC showed that the concordance of the two methods was highest in the 3+ group (100%) and lower in 1+ group (89%), whereas a high degree of discordance was found in the 2+ group (69%). Conclusions. CISH is an accurate and practical technique for the evaluation of both HER2 gene and chromosome 17 status and its application is considered necessary especially for the clarification of the 2+ results of IHC.

HER2 is a gene located on the long arm of chromosome 17 (17q12-21.32). The encoded product is a transmembrane 185 kDa protein with tyrosine kinase activity that belongs to the type I epidermal growth factor receptor family (1) and is known to be involved in the signal transduction of cell growth (2, 3). Amplification of the gene and concomitant overexpression of the HER2 protein has been identified in approximately 10-34% of breast carcinomas and is considered to be not only an important biological marker of poor prognosis but also a useful marker for the response to chemotherapy or endocrine therapy (2, 4). The development of the new anticancer drug trastuzumab (a recombinant humanized monoclonal antibody against the extracellular part of the HER2 protein) and the encouraging results obtained from the treatment of patients whose breast carcinomas demonstrate HER2 amplification and/or overexpression have renewed the interest in HER2. Therefore, laboratory assessment of HER2 gene status has become a basic procedure for the appropriate management of breast cancer patients (5).

The earliest studies of HER2 used solid matrix blotting techniques for the detection of gene amplification (southern blots) and protein overexpression (western blots). These methodologies are too complicated for routine diagnostics and are also affected by the ratio of tumor to stromal elements which is particularly significant in breast cancer because nonmalignant cells might constitute more than 50% of the cells in a given sample. For this reason the above techniques are currently replaced by fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC). The immunohistochemical technique has been mainly used in the pathology lab in a routine setting for the detection of HER2 overexpression on archival material from breast carcinomas. However immunohistochemical examination is subject to technical effects which are related to fixation and

Correspondence to: Nikiforos Kapranos, MD, PhD, MITERA Maternity and Surgical Center, Department of Pathology, 6 Erythrou Stavrou Str., 151 23 Athens, Greece. Tel: (+301) 210-6869134, Fax:(+301) 210-6462686, e-mail: nkapranos@mitera.gr

Key Words: Breast cancer, chromogenic in situ hybridization, HER2, chromosome 17 polysomy, immunohistochemistry.
methodological differences such as the type of antibody and the scoring system used (4). Since the ability to immunohistochemically detect the HER2 protein depends on the presence of oncogene amplification, FISH analysis, which defines the number of gene copies in the cancer cell nucleus, started to be alternatively or additionally used for the evaluation of HER2 status. The FISH technique, on the other hand, requires expensive fluorescence microscopy with all the concomitant disadvantages of evaluating and retaining the hybridization signal. For this purpose a new technique, chromogenic in situ hybridization (CISH) was developed in which the DNA probe is detected using a simple immunohistochemical-like peroxidase reaction and the signal is evaluated by a simple optical microscope (6).

The purpose of this study was to evaluate HER2 gene status in relation to chromosome 17 polysomy with the CISH technique and to compare the results with those of IHC.

Materials and Methods

Sixty six cases of invasive ductal breast carcinoma from patients 39-79 y.o. who underwent mastectomy in Helena Venizelou Hospital were included in this study. The selection process was based on the results of HER2 IHC over a period of one year. All the cases included showed some type of HER2 immunoreactivity (1+, 2+, 3+). The 2+ cases were overrepresented (26/66 cases) since these constitute a controversial group that requires confirmatory testing for reliable determination of HER2 positivity and thus the most possible candidate for the application of CISH technique. The exclusion of all immuno-negative cases was based on the previous finding that none of these had HER2 gene amplification (7).

Immunohistochemistry. Tumor sections of 4 µm were mounted on siliconized glass slides, air dried and heated at 45°C overnight. After deparaffinization and rehydration the sections were incubated in 0.01M citrate buffer in a microwave oven for 15 min for antigen retrieval. After blocking for nonspecific antibody binding, the CB-11 monoclonal antibody at a dilution 1:50 (Monosan, Uden, The Netherlands) was applied. Signal visualization was performed by the avidin-biotin-peroxidase method using DAB as chromogen and hematoxylin as counterstain. As negative control we used tumor sections in which normal horse serum was used instead of the primary antibody.

Evaluation of HER2 immunohistochemical expression was performed according to the scoring guidelines of the HercepTest™ as follows: Score 0: no staining or membrane staining in <10% of tumor cells; Score 1+: faint membrane staining in >10% of tumor cells; Score 2+: weak – moderate complete membrane staining in >10% of tumor cells and 3+: strong, complete membrane staining in >10% of tumor cells. Scores of 0 and 1+ were considered as negative for HER2 expression while 2+ and 3+ as immunopositive.

Chromogenic in situ hybridization. CISH was performed on 5-µm thick formalin-fixed paraffin-embedded tissue sections. In brief, the sections were deparaffinized and incubated in Tris-EDTA buffer within a microwave oven at 96°-100°C for 10 minutes. Subsequently the sections were treated enzymatically using pepsin at 37°C for 3-6 min, washed in PBS and dehydrated in graded dilutions of ethanol. Ten µl ready to use digoxigenin-labeled HER2 probe (Zymed, San Francisco, CA, USA) was applied to the slide and covered with a coverslip. The slides were denatured on a hot plate (96°C for 5 min) and the hybridization reaction was subsequently performed overnight at 37°C. After hybridization, the slides were washed at 75°C for 5 min and immersed in absolute methanol with 3% H2O2. Detection of hybridization reaction was carried out using the Spot-Light CISH polymer detection Kit (Zymed), as follows: Sequential incubation of the sections with mouse anti-digoxigenin and Polymerized HRP Goat anti-Mouse antibody, and mounted. Serial tumor sections in which PBS was used instead of HER2 probe were used as negative controls.

The CISH signals were evaluated using a Nikon E600 (Nikon, Tokyo, Japan) microscope equipped with 40X and 60X objective lenses. Cases with 1-5 gene copies per nucleus were defined as negative for amplification; detection of 6-10 gene copies per nucleus in >50% of tumor cells were defined as low level amplification (LLA) and 11-20 gene copies per nucleus and/or a large gene copy cluster in >50% of tumor cells as high level amplification (HLA) of HER2. A case of breast carcinoma, with 3+ immunohistochemical HER-2 protein expression showing HLA (Monosan, Uden, The Netherlands) was applied. Signal visualization was performed by the avidin-biotin-peroxidase method using DAB as chromogen and hematoxylin as counterstain. As negative control we used tumor sections in which normal horse serum was used instead of the primary antibody.

Evaluation of HER2 immunohistochemical expression was performed according to the scoring guidelines of the HercepTest™ as follows: Score 0: no staining or membrane staining in <10% of tumor cells; Score 1+: faint membrane staining in >10% of tumor cells; Score 2+: weak – moderate complete membrane staining in >10% of tumor cells and 3+: strong, complete membrane staining in >10% of tumor cells. Scores of 0 and 1+ were considered as negative for HER2 expression while 2+ and 3+ as immunopositive.

Chromogenic in situ hybridization. CISH was performed on 5-µm thick formalin-fixed paraffin-embedded tissue sections. In brief, the sections were deparaffinized and incubated in Tris-EDTA buffer within a microwave oven at 96°-100°C for 10 minutes. Subsequently the sections were treated enzymatically using pepsin at 37°C for 3-6 min, washed in PBS and dehydrated in graded dilutions of ethanol. Ten µl ready to use digoxigenin-labeled HER2 probe (Zymed, San Francisco, CA, USA) was applied to the slide and covered with a coverslip. The slides were denatured on a hot plate (96°C for 5 min) and the hybridization reaction was subsequently performed overnight at 37°C. After hybridization, the slides were washed at 75°C for 5 min and immersed in absolute methanol with 3% H2O2. Detection of hybridization reaction was carried out using the Spot-Light CISH polymer detection Kit (Zymed), as follows: Sequential incubation of the sections with mouse anti-digoxigenin and Polymerized HRP Goat anti-Mouse antibody, and mounted. Serial tumor sections in which PBS was used instead of HER2 probe were used as negative controls.

The CISH signals were evaluated using a Nikon E600 (Nikon, Tokyo, Japan) microscope equipped with 40X and 60X objective lenses. Cases with 1-5 gene copies per nucleus were defined as negative for amplification; detection of 6-10 gene copies per nucleus in >50% of tumor cells were defined as low level amplification (LLA) and 11-20 gene copies per nucleus and/or a large gene copy cluster in >50% of tumor cells as high level amplification (HLA) of HER2. A case of breast carcinoma, with 3+ immunohistochemical HER-2 protein expression showing HLA by CISH was used as a positive control. Serial tumor sections in which PBS was used instead of HER2/neu probe were used as negative controls.

In order to confirm that the increased number of HER2 gene copies were due to amplification and not due to chromosome 17 polysomy, additional CISH analysis with a biotin-labeled chromosome 17 centromeric probe was applied on adjacent sections in all cases with LLA. Chromosome 17 CISH analysis was performed according to ZYMED’s protocol for formalin fixed paraffin embedded tissue sections.

Results

CISH detection for HER2 amplification in the 66 tumors examined was classified as follows: Thirty four (51.5%) with no amplification, 10 (15%) with LLA and 22 (33.5%) with HLA. From the 22 cases with HLA, 4 showed multiple gene copies (10-20), 5 showed large gene copy clusters (Figure 1)
and the remaining 13 a combination of both the above types of gene amplification. The comparison of the results obtained by CISH and IHC are summarized in Table I. As it can be seen the concordance of the results of the two methods was high in the 3+ group and lower in the 1+ group, whereas a high degree of discordance was found in the 2+ group. In this group 18 of the 26 (69%) immunohistochemically positive cases were negative for gene amplification (Figure 2).

The results of CISH analysis in the 10 cases with LLA is summarized in Table II. As can be seen in case 1 the ratio of HER2 to chromosome 17 hybridization signals was lower than 2 while in all the other cases the ratio was equal or greater than 2. Specifically, in case 1 the number of chromosome 17 copies was 4-7 per nucleus (Figure 3) whereas in all other cases the number ranged from 2 to 5 copies per nucleus (Figure 4).

Discussion

The two most commonly used techniques for the analysis of HER2 status are the assessment of protein overexpression by IHC and the evaluation of gene amplification by FISH. However, there are advantages and disadvantages to each methodology (7-9). Specifically immunohistochemical analysis is relatively inexpensive and fast, it doesn’t demand specialized laboratory equipment and most importantly it directly determines the HER2 protein since treatment with Herceptin is specifically targeted toward the extracellular component of the HER2 protein on the cell surface. However the quantification of protein overexpression is significantly affected by a number of factors such as tissue fixation and processing, antigen retrieval, the sensitivity and specificity of the numerous commercially available anti HER2 antibodies as well as the various scoring systems used. FISH has been alternatively or additionally used for the detection of HER2 gene alterations. This technique is less vulnerable to the above related to tissue processing factors, since DNA, the target of FISH, is more stable than the HER2 protein. Furthermore the results are much less subjective than those of IHC as they concern the enumeration of fluorescent signals rather than the evaluation of staining intensity. Finally the threshold for positivity has been standardized in contrast to IHC where there is no uniformly accepted cut-off point for positivity. In a recent study addressing the accuracy and reproducibility of HER2 assays it was shown that IHC-based tests were more susceptible to interobserver variation (K=0.67) than FISH (K=0.973) (10). However FISH has also important disadvantages such as the temporarity of the fluorescence signals which fade within a few weeks and the use of expensive equipment (epifluorescence microscope, special filters, digital camera) which do not allow the application of this method in most routine diagnostic laboratories.

Recently an alternative method, CISH was developed, which combines the advantages of both IHC and FISH (6). This approach enables the identification and quantification of HER2 gene copies with a conventional peroxidase reaction visualized with usual optical bright field microscopy rather than fluorescence as in FISH. This method seems to incorporate many and significant advantages over FISH. Specifically, it can be reliably applied on paraffin sections, it is much less expensive than FISH, as it doesn’t demand the use of complicated laboratory equipment associated with fluorescence microscopy. Moreover, the time and effort needed for the evaluation of CISH is much shorter than for FISH and the signal remains stable over time. Finally CISH permits the easy correlation of the signals with morphologic features of the specimens, a fact particularly significant in cases with extensive DCIS elements. Specifically, it has been reported that in some of these tumors although the DCIS component showed 3+ membrane HER2 staining, the invasive component showed membrane staining of lower intensity (2+) or was even negative (1+, 0) (11).

According to the literature a few studies have compared the results obtained by FISH and CISH. In the first study by Tanner et al. (6) a very good rate of concordance was reported in a series of 157 breast cancers (kappa coefficient: 0.81). The few differences reported were most likely attributed to the different sample materials (FISH: cell imprints, CISH:paraffin sections). In the study of Zhao et al. (12) a concordance rate of 100% was found although this was not surprising since the same probe used in the CISH assay was first labeled with fluorescence and then relabeled with a peroxidase-diaminobenzidine detection system. A similar

---

Table II. Chromosome 17 CISH analysis in 10 immunopositive tumors with low level HER2 amplification.

<table>
<thead>
<tr>
<th>Case nr</th>
<th>IHC</th>
<th>HER2 CISH</th>
<th>Chr17 CISH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3+</td>
<td>6-8</td>
<td>4-7</td>
</tr>
<tr>
<td>2</td>
<td>3+</td>
<td>6-10</td>
<td>2-4</td>
</tr>
<tr>
<td>3</td>
<td>3+</td>
<td>8-10</td>
<td>2-5</td>
</tr>
<tr>
<td>4</td>
<td>3+</td>
<td>7-9</td>
<td>2-4</td>
</tr>
<tr>
<td>5</td>
<td>3+</td>
<td>8-10</td>
<td>2-3</td>
</tr>
<tr>
<td>6</td>
<td>3+</td>
<td>7-10</td>
<td>2-4</td>
</tr>
<tr>
<td>7</td>
<td>2+</td>
<td>6-10</td>
<td>2-3</td>
</tr>
<tr>
<td>8</td>
<td>2+</td>
<td>6-8</td>
<td>2-3</td>
</tr>
<tr>
<td>9</td>
<td>2+</td>
<td>6-10</td>
<td>2-4</td>
</tr>
<tr>
<td>10</td>
<td>2+</td>
<td>7-9</td>
<td>2-3</td>
</tr>
</tbody>
</table>
Figure 1. HER2 IHC staining and CISH in breast carcinoma: Tumor with 3+ IHC staining (A) showing HER2 gene amplification by CISH appearing as large gene clusters (B). Original magnification 400X

Figure 2. HER2 IHC staining and CISH in breast carcinoma: Tumor with 2+ IHC (A) showing the absence of HER2 gene amplification with mostly two to three signals per nucleus (B). Original magnification 600X
Figure 3. Chromosome 17 CISH in breast carcinoma: Tumor with 3+ HER2 IHC staining and LLA of HER2 gene by CISH showing 4-7 signals per nucleus. Original magnification 600X

Figure 4. Chromosome 17 CISH in breast carcinoma: Tumor with 2+ HER2 IHC staining and LLA of HER2 gene by CISH showing 2-4 signals per nucleus. Original magnification 400X
concordance rate (100%) was also found in the study of Dandachi et al. (13) while other two recent studies reported concordance rates of 84% and 96% respectively (14, 15). Finally most recently Park et al. (16) investigated the correlation between CISH and FISH in 188 breast carcinomas using tissue microarray technology and reported a very high concordance rate (94.1%) between the two assays.

In the present study all the tumors classified immunohistochemically in the 3+ group were found to have either high or low level amplification of the HER2 gene while the immunohistochemical groups 1+ and 2+ showed a discordance rate of 11% and 69% respectively. These findings are in agreement with most of other studies in the literature (11, 17-22). However, in the recent publication of Hammock et al. a 51% discordance rate between strong 3+ protein expression and gene amplification was demonstrated (23). It is important to notice that both the results of published studies (17, 24-30) as well as our findings (Table III) emphasize the significant issue of the poor correlation between IHC and FISH-CISH in the subset of 2+ HER2 immunopositive tumors. Based on these we also believe that additional HER2 analysis for the evaluation of gene amplification is necessary for these tumors and the results of both assays should be considered before making a decision for anti HER2 therapy.

According to our findings of the 32 tumors showing gene amplification, the 10 had low and the 22 high level amplification. Xing et al. (31) were the first to make a similar distinction. In their study the authors identified a subset of patients with what they defined as "low grade amplification" and demonstrated that this group had a significantly lower incidence of recurrence compared to the cases with high-grade amplification. The additional application of CISH for chromosome 17 on all 6 cases with 3+ HER2 immunopositivity and LLA revealed that one of these had a similar number of copies of HER2 and chromosome 17 confirming the absence of conventional amplification and the presence of a rather high level of polysomy (Table II). It has been reported that occasional breast carcinomas with HER2 3+ immunostaining may be negative for gene amplification by FISH according to standard scoring criteria (24, 27, 32). This finding was attributed mainly to two factors, false positive immunostaining or protein overexpression without gene amplification. Recent articles provide evidence of a third factor, namely chromosome 17 polysomy. These studies (15, 28, 29, 33) have addressed the important issue of the role and impact of chromosome 17 polysomy in a subset of immunopositive tumors showing borderline HER2 amplification. Wang et al. (33) concluded that aneusomy of chromosome 17 is common in invasive breast cancer specimens (51.3%) and that cases with high polysomy 17 (≥3.56 signal/nucleus), all of which contained concurrent modest HER2 amplification may have an additive affect on gene dosage and protein expression. In the very interesting study of Lal et al. (29) it was reported that IHC 3+ immunostaining without scorable gene amplification may indeed be, at least in some cases, the result of increased HER2 protein expression secondary to an pathetically increased total number of HER2 gene copies per tumor cell. They suggested that it would be reasonable to examine whether these tumors are biologically distinct from other FISH negative tumors and more similar to tumors with conventional HER2 amplification, especially in terms of response to Herceptin based therapy. The additional application of CISH for chromosome 17 on all four cases with 2+ HER2 immunopositivity and LLA demonstrated that all had an equal or larger than two ratio of HER2 to chromosome 17 hybridization signals (Table II). Perez et al. (28) observed an incidence of extra copies of HER2 relative to chromosome 17 in 25% of their specimens with a 2+ Herceptest result. They preferred to interpret these as

<table>
<thead>
<tr>
<th>Study</th>
<th>Nr of cases</th>
<th>IHC</th>
<th>FISH</th>
<th>CISH</th>
<th>No (%) amplified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass et al. (24)</td>
<td>96</td>
<td>CTA</td>
<td>Pathvysion</td>
<td>ND*</td>
<td>21 (24)</td>
</tr>
<tr>
<td>Starr et al. (25)</td>
<td>18</td>
<td>Herceptest</td>
<td>Pathvysion</td>
<td>ND</td>
<td>5 (28)</td>
</tr>
<tr>
<td>Buehler et al. (26)</td>
<td>72</td>
<td>Herceptest</td>
<td>Inform kit</td>
<td>ND</td>
<td>22 (30)</td>
</tr>
<tr>
<td>Kakar et al. (17)</td>
<td>17</td>
<td>CB11</td>
<td>Pathvysion</td>
<td>ND</td>
<td>6 (35)</td>
</tr>
<tr>
<td>Tubbs et al. (27)</td>
<td>41</td>
<td>Herceptest/CB11</td>
<td>Inform kit</td>
<td>ND</td>
<td>7 (17)</td>
</tr>
<tr>
<td>Perez et al. (28)</td>
<td>216</td>
<td>Herceptest</td>
<td>Pathvysion</td>
<td>ND</td>
<td>26 (12)</td>
</tr>
<tr>
<td>Lal et al. (29)</td>
<td>63</td>
<td>Herceptest</td>
<td>Pathvysion</td>
<td>Zymed</td>
<td>20 (22)</td>
</tr>
<tr>
<td>Kobayashi et al. (30)</td>
<td>27</td>
<td>Nitirei</td>
<td>Pathvysion</td>
<td>ND</td>
<td>5 (18.5)</td>
</tr>
<tr>
<td>Present study</td>
<td>26</td>
<td>CB11</td>
<td>ND</td>
<td>Zymed</td>
<td>8 (31)</td>
</tr>
</tbody>
</table>

* Not done
having HER2 duplication rather than low level polysomy and suggested that duplication of HER2 may be associated with HER2 protein overexpression and emphasized the necessity of clinical trials in order to determine whether patients with HER2 duplication (or LLA) should be considered for trastuzumab therapy.

In conclusion, CISH seems to be a specific, sensitive and easily applicable technique for the detection of both HER2 gene amplification and chromosome 17 status. The advantages of this technique in relation to recent reports that show high concordance between CISH and FISH, indicate that CISH may be the method of choice for the investigation of HER2 gene status in the routine practice of pathology laboratories. The application of this technique is especially useful for the clarification of the 2+ results of the immunohistochemical staining as well as for the confirmation of amplification in cases with 6-10 HER2 gene copies. Therefore the combined use of IHC and CISH may be useful for the better selection of patients with breast cancer that will benefit from therapy with Herceptin.

Acknowledgements

The authors thank Dr Harry Petropoulos, Bioland Inc. for his technical and financial support.

References


Received August 30, 2004
Accepted January 21, 2005