# RESEARCH ARTICLE

# Chromogenic In Situ Hybridisation Test for Breast Cancer Patients with Equivocal IHC Results - a Study from Iran

Mitra Mehrazma[1], Elham Kalantari[2], Hamid Rezvani[3], Babak Bahar[4], Ali Basi[5], Seyed Mohsen Razavi[5], Nasser Rakhshani[6]*

## Abstract

Background: HER2/neu overexpression on cell membranes of breast cancer cells is due to HER2/neu gene amplification and it is important to identify potential candidates for anti HER2 therapy with trastuzumab. IHC, FISH and CISH are standard FDA approved assays currently used to determine HER2 status in routine practice. The aim of this study was to determine HER2 gene amplification, using the CISH method in breast carcinoma samples which had IHC +2 reactions. Materials and Methods: This study was conducted from 2008-2010 using 334 consecutive breast carcinoma samples referred from local laboratories to Mehr Hospital. CISH assays were performed for all cases, and IHC tests were also done for determining efficacy and accuracy of local labs. HER2 status in local IHC tests was compared with central IHC and CISH results. Results: Of 334 breast cancer patients, 16 were negative for HER2 IHC (0, +1), 201 cases were equivocal (+2), and 31 positive (+3). Of 334 referral cases, 88 were CISH positive (26.3%) and 246 were CISH negative (73.7%). Of 201 IHC +2 cases, HER2 gene amplification was observed in 42 cases (kappa: 0.42). A 29.9% concordance was found between local IHC and central IHC. Sensitivity and specificity of local IHC were 90% and 53.8%, respectively. Conclusions: Low accuracy of IHC results in local labs was associated with the following factors: using former FDA-approved criteria for HER2 interpretation, utilizing non-validated kits, and lack of any quality assurance program. Therefore, following the new 2014 ASCO/CAP guideline and comprehensive quality assurance should be implemented to ensure accuracy of HER2 testing.

Keywords: Breast cancer - CISH - IHC - HER-2/neu - Iran

## Introduction

The most widely studied biomarker among various types of breast cancer prognostic biomarkers is the human epidermal growth factor receptor 2 HER2 gene (Wolff et al., 2013). Approximately, 15-30% of breast cancers show amplification in HER2 gene, leading to poor prognosis in patients with breast cancers (Piccart et al., 2002; Chang et al., 2004; Yarden et al., 2004). Amplification of this gene is associated with various clinical effects including disease progression, increased metastatic potential, shorter survival, higher rates of relapse, high-grade tumors, resistance to Tamoxifen therapy, and surprisingly good response to trastuzumab targeted therapy (Ferrero-Pous et al., 2000; Ariga et al., 2005; Huang et al., 2005). Targeted-therapy using monoclonal antibody against HER2, trastuzumab (Herceptin) and the dual tyrosine kinase inhibitor (HER1 and HER2)-Lapatinib is an effective treatment for patients with HER2 gene amplification (Slamon et al., 2001; Yarden et al., 2004; Bilancia et al., 2007). But, both drugs have been found to be effective only in tumors showing true gene amplification. So, it is important to find patients who may respond well to targeted therapy. To detect HER2 status, following methods are used: a) gene analysis (Chromogenic in situ hybridization (CISH), Fluorescent in situ hybridization (FISH), Polymerase chain reaction (PCR), Southern blot, and detection of messenger RNA by RT-PCR or Northern blot), b) direct detection of HER2 protein on the cell membrane by western blot and immunohistochemistry (IHC). IHC, FISH and CISH are the most common methods to determine HER2 status (Ivkovic-Kapicl et al., 2007). HER2 protein can be stained by various immunohistochemical methods; Ventana pathway and Hercep Test are FDA-approved kits for the assessment of HER2 gene by IHC (O'Malley et al., 2008). PathVysion and Invitrogen are FDA-approved kits for HER2 gene detection by FISH and CISH, respectively. IHC is less expensive and more widely available but has poorer reliability (Lan et al., 2005). This has led to debate about the best testing strategy, fueled by the high cost of the drug. The ASCO/CAP study data from 2000 to 2005

*[1]Oncopathology Research Center and Department of Pathology, [2]Oncopathology Research Center, [5]Department of Oncology, [6]Department of Pathology, GILDRC, FiroozgarHospital, Iran University of Medical sciences, [3]Department of Oncology, Shahid Beheshti University of Medical Sciences, [4]Department of Oncology Shariati Hospital Tehran University of Medical Sciences, Tehran, Iran  *For correspondence: ek13720@yahoo.com*

jointly with adjuvant randomized trials, demonstrated that as many as 15% to 20% of the HER2 assays performed in the field may be incorrect when the same specimens were re-evaluated in a high-value central laboratory (Wolff et al., 2006). Regarding the high-rate of false-positive IHC results based on original FDA-approved criteria, breast cancer's high cost of treatment, and Trastuzumab-related cardiotoxicity, the College of American Pathologists (CAP) and the American Society of Clinical Oncology (ASCO) provided guidelines for HER2 testing (Wolff et al., 2006). These updated guideline recommendations were published in 2007 to avoid number of false positive IHC cases (Vogel, 2010). Equivocal IHC results (+2) can be seen in 15% of total breast cancers, of whom 24%-44% show amplification (Lan et al., 2005; Wolff et al., 2006). Although the results obtained using IHC, FISH and CISH methods show 90-95% concordance, the primary differences are appeared to be due to (+2) IHC results. So, most guidelines recommend testing all IHC+2 samples with FISH or CISH (Wolff et al., 2006). The aims of this paper are to determine the frequency of amplifications in the study group and to examine the differences between the results of this study and findings obtained in other studies. Finally we explain how far we are from ASCO/CAP new guideline.

## Materials and Methods

The purpose of this experimental trial is to detect HER2 biomarker in tissue samples of 334 patients with breast cancer who were referred to Pathology department of Mehr hospital, since the implementation of CISH technique, between March 2009 and Feb 2011. This study used dual CISH as the gold standard method to determine the accuracy of the IHC test results. In brief, IHC and CISH tests performed in Mehr laboratory are called central tests while IHC tests performed in other laboratories are called local IHC tests. Central laboratory IHC staining test has been validated by FISH test and the quality of CISH technique has been comprehensively controlled by NEQAS. The study is looking at tissue samples that were collected from women referred to this center with a primary diagnosis of breast cancer. Tissue samples were divided into two groups: a) First group included IHC+2 cases referred to CISH. Actually, local IHC results were available in this group of samples. b) Second group consisted of samples in which local IHC results were not available and just referred for CISH test. CISH test along with IHC staining were carried out in both group of tissue samples to evaluate the status of target population (IHC+2) and assess performance of laboratories for accuracy and precision.

### Test methods
Paraffin blocks were cut into 5-6 micron sections (at least 2 sections) in order to evaluate the HER2 marker. All original breast tumor tissues with either modified radical mastectomy or breast conserving surgery were sectioned to confirm the diagnosis of invasive carcinomas

### Chromogenic in situ hybridization

The test has been performed by the use of chromogenic in-situ hybridization, according to Zyto Dot® 2C SPEC HER2/CEN 17 dual Probes Kit protocol. (Zytovision company-Germany) The PD-12 probe contains digoxigenin-labled polynucleotides, which target sequences of the HER2 gene and DNA-labeled polynucleiotides, which target alpha-satellites of the centromere of chromosome 17 led to the formation of green and red signals that can be visualized by light microscopy using a 40x objective. All of these reactions were carried out within 2 days and following 4 steps according to kit protocol (www.zytovision.com).

The CISH hybridization signal of one single copy of a HER2 gene appears as a dark green-colored distinct dot-shaped signal, while the signal of one single copy of a chromosome 17 centromeric region appears as bright red-colored distinct dot-shaped signal which can be clearly distinguished from the background counterstained with hematoxylin. All slides were reviewed by two experienced pathologists and results were recorded and scored according to the new 2014 ASCO/CAP guidelines. In brief, the number of CEP17 and HER2 signals counted in 20 non-overlapping invasive cancer cell nuclei, using at least three distinct tumor fields (when possible). HER2 signal heterogeneity was disregarded from this study. Where the mean HER2/CEP17 ratio in any field is 2 or greater, the tumor regarded as amplified and ratio less than 2 regarded as not amplified when her2 copy number is equal or less than 4. Cases with ratio less than 2 and her2 copy number between 4-6 regarded as equivocal borderline results and final decision on the degree of amplification was made after counting an additional 20 nuclei according to new 2014 ASCO/CAP guideline(Wolff et al., 2013).

### Immunohistochemistry
IHC staining was performed on paraffin-embedded breast cancer tissue specimens using polyclonal rabbit antihuman c-erbB-2 oncoprotein (Dako A0485 clone). IHC staining procedure was conducted according to Dako Envision kit protocol in the following steps. Finally, the slide was counterstained with hematoxylin and reviewed by two pathologists under light microscope at 40x magnification. IHC results were recorded and scored according to the new 2014 ASCO/CAP guidelines (Wolff et al., 2013). In brief Positive staining, i.e. "*3+*", was defined as strong, complete, homogeneous membrane staining ("*chicken-wire*" pattern) in >10% of cells. Equivocal staining, i.e. "*2+*", was assessed as moderate/strong, incomplete membrane staining in less than 10% of cells. No staining, i.e. "*0*", or weak, incomplete membrane staining, i.e. "*1+*", in any percentage of cells were categorized as negative.

### Statistical analysis
After data collection, frequency tables, graphs and statistics were used to summarize and organize data. Chi-square and McNemar's tests were used for statistical analysis. Sensitivity and specificity of local and central IHC tests, and finally the concordance rate between local and central IHC tests were determined. Concordance calculation performed based on compatibility rate

between IHC and CISH on all positive and negative cases, and finally between local and central laboratory IHC results, when the overall percent agreement is similar. Kappa values are interpreted as follows: 0-0.20, poor agreement, 0.21- 0.45, moderate agreement, 0.46-0.75, high agreement and 0.76-0.99, almost perfect agreement. The identity of patients is not revealed in this study.

## Results

Of 337 cases, 1 case identified to have chromosome 17 polysomy and 2 cases with equivocal CISH results were discontinued from the study, so data analysis was only performed on the remaining 334 breast cancer specimens. Based on local and central IHC method and scoring system, the frequency distribution of HER2 protein among cases referred to Mehr laboratory has been shown in Table1. Among of 334 cases, 88 (26.3%) showed HER2 gene amplification by CISH and 246 (73.7%) showed no amplification. The frequency of HER2 gene amplification by CISH method between local IHC2+ and central IHC2+ are 20.9% and 26.3%, respectively (figure1). The following figure shows frequency of HER2 gene amplification.

*Comparison between local IHC and CISH*

14 out of 16 (87.5%) samples that were reported negative by local IHC (0 or +1), were also CISH negative and among 30 cases scored as IHC positive (+3), only 18 cases (60%) exhibited HER2 gene amplification. The

results using local IHC and CISH for IHC (0, +1, and +3) samples showed a concordance of 69.6%. The comparison between results reported by local HER2 IHC test ( without considering IHC+2) and CISH revealed that there was a significant correlation between HER2 score in breast cancer specimens by local IHC and CISH (P=0.002). IHC sensitivity and specificity were determined 90% and 53.8%, respectively and statistical analysis showed moderate concordance between local testing with IHC and CISH results (Kappa= 0.42).

*Comparison between central IHC and CISH*

These analyses were also performed for central laboratory HER2 IHC testing results. According to findings, among 177 cases that were reported negative (IHC+1, 0) by central IHC, 169 cases (95.5%) remained negative on the CISH test (without amplification) and among of 62 central IHC-positive cases, 55 (87.7%) remained positive on the CISH test. The concordance between central IHC and CISH for all positive and negative cases (IHC+3, +1 and 0) were reported 93.7%.

**Table 1. The Frequency of HER2 Status by IHC Method**

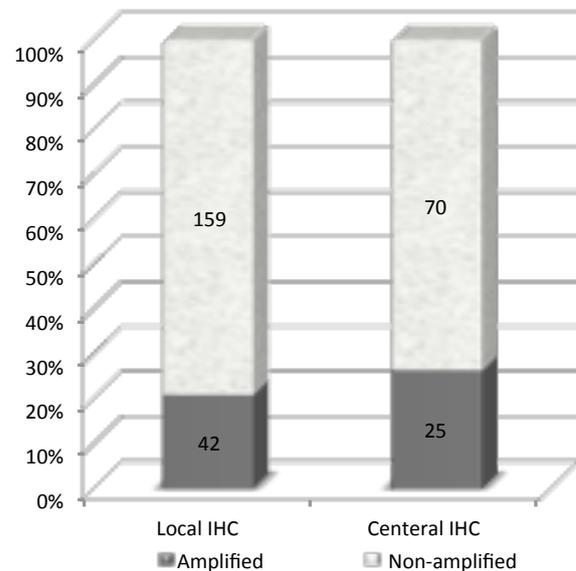| Grade | IHC local | | IHC central | |
|---|---|---|---|---|
| | Number | Percent | Number | Percent |
| 0, +1 | 16 | 4.8 | 177 | 53 |
| 2 | 201 | 60.1 | 95 | 28.4 |
| 3 | 30 | 8.9 | 62 | 18.6 |
| Not graded | 88 | 26.2 | 0 | 0 |
| Total | 334 | 100 | 334 | 100 |

*IHC: Immuno-Histo-Chemistry



**Figure 1. The Frequency of HER2 Status by IHC Method**

**Table 2. Local and Central IHC Characteristics in Comparison with CISH as a Gold Standard Method**

| | Sensitivity | Specificity Concordance | IHC+3 | IHC 0, +1, Concordance | IHC 0, +1, +3 Concordance | Discordant results* | Concordance rate with CISH** | Kappa value |
|---|---|---|---|---|---|---|---|---|
| Local IHC | 90% | 53.80% | 60% | 87.50% | 69.60% | 30% | Moderate | 0.42 |
| Central IHC | 87.30% | 96% | 88.70% | 95.50% | 93.70% | 6.30% | Almost perfect | 0.84 |

*Discordant Results are related to IHC +3 cases not showing amplification by CISH and vice versa, ** Chromogenic in-situ hybridization

**Table 3. Retested Central IHC Results for Referral HER2**

| Local IHC | 0 and +1 | | +2 | | +3 | | Totally | |
|---|---|---|---|---|---|---|---|---|
| Central IHC | Number | Percent | Number | Percent | Number | Percent | Number | Percent |
| +1and 0 | 10 | 62.5 | 5 | 31.25 | 1 | 6.25 | 16 | 100 |
| +2 | 121 | 60.19 | 51 | 25.37 | 29 | 14.42 | 201 | 100 |
| +3 | 4 | 13.33 | 13 | 43.33 | 13 | 43.33 | 30 | 100 |
| Totally | 135 | 54.65 | 69 | 27.93 | 43 | 17.4 | 247 | 100 |

**Table 4. The comparison between IHC and in situ hybridization tests(FISH or CISH) in various studies**

| Study | | Positive Concordance | Negative Concordance | Overall Concordance |
|---|---|---|---|---|
| O'Malley | | 0.861 | 0.985 | - |
| Roche | | - | - | 0.66 |
| Madrid | | 0.725 | 1 | 0.862 |
| Laakso | | - | - | 0.91 |
| Panjwani | | 0.939 | 0.859 | - |
| Ridolfi | | 1 | 0.982 | 0.89 |
| Dandachi | | 0.917 | 1 | 0.955 |
| ASCO/CAP guidline | | 0.9 | 0.95 | 0.95 |
| Local | Our Study | 0.6 | 0.875 | 0.696 |
| Central | | 0.887 | 0.955 | 0.937 |

Another analysis showed that there was almost perfect concordance between central IHC and CISH results (Kappa=0.84). Sensitivity and specificity of central IHC were 87.3% and 96%, respectively (Table2).

*Comparison of local IHC result with central IHC result*

The comparison between the results of IHC cases retested centrally to determine HER2 status and local IHC (+3, +2, and 0) showed that there was statistically significant difference between two groups(P<0.0001). But, there was actually no significant difference when IHC+2 cases were excluded from the study (P=0.37). The findings show that large differences between local and central IHC assays are due to IHC+2 cases, and all cases scored as IHC positive (+3) and negative (+1, 0) have relatively similar results (Table3). The total concordance between local and central IHC was 29.9% and the highest rate of discordance was between the results of local IHC +2 and central IHC+2 (74.6%).

## Discussion

The advances in prognostic factors and therapeutic options for human breast cancer have resulted in finding more accurate methods to assess the HER2 status. In many studies, IHC has been established as first test to detect HER2 over expression on the surface of tumor cells. IHC method with all the advantages of wide availability and low cost that make it preferred first test, owing to technical difficulties and interobservation variation among pathologists in interpretation may provide misleading results. Thomson et al wrote that IHC staining cannot be adopted for cases scored as 2 in routine clinical practice and supplementary methods like FISH should be used as well (Thomson et al., 2001). Kakar et al. (2000) concluded that IHC staining is an appropriate method for the assessment of HER2 status but FISH method should be performed for cases scored less than +3 (especially +2 by IHC) to achieve better results (Kakar et al., 2000). CISH, which is a new development in the detection of HER2, is well-suited for use as an alternative to FISH (Tanner et al., 2000; Kumamoto et al., 2001; Dandachi et

al., 2002; Zhao et al., 2002; Arnould et al., 2003; Gupta et al., 2003; Park et al., 2003; Madrid and Lo, 2004; Vera-Roman and Rubio-Martinez, 2004). CISH method has several advantages over FISH, including the use of ordinary microscope; the method is less cumbersome and cost effective, as well as the signal intensity is permanent. The concordance between CISH and FISH ranged from 85% to as high as 100%. The first study on CISH method was conducted by Taner et al. in 2000 followed by several researches on the priority of CISH over previous methods including FISH (Tanner et al., 2000).

The results of the study conducted by Madrid et al. on 160 cases using CISH and its comparison with IHC staining showed that CISH due to high accuracy and diagnostic yields in can play a complementary test in cases with equivocal IHC status (Madrid and Lo, 2004). By 2005, "Bargava" et al. (2005) reported that both CISH and FISH have the same result of HER2 gene amplification in human breast cancer (Bhargava et al., 2005). The results of the study conducted by Lakso et al using CISH and FISH along with Dandachi et al study on 173 breast cancer specimens using IHC and CISH showed the perfect concordance (k=0.82 and 0.88, respectively) (Dandachi et al., 2002; Laakso et al., 2006). Concordance between IHC and ISH has been reviewed in several studies and the results are compared with the present study and summarized in Table4.

Our analysis showed that 26.3% of all specimens irrespective of central or local IHC result had HER2 gene amplification by CISH which is in expected range of 15% to 30% mentioned in most studies (Piccart et al., 2002; Chang et al., 2004; Yarden et al., 2004). From 201 IHC 2+ cases referred to our center revealed that 20.9% of them had amplification by CISH, which is lower than expected range of 24% to 44% seen in most studies (Lan et al., 2005; Wolff et al., 2006). Amplification rate seen in central IHC+2 cases was found to be 26.3% which was within the expected range

Discordant Results: 3+IHC cases that are FISH non-amplified and IHC-negative cases that are FISH-amplified contain 1.5%-19.2% of all cases in the studies (Reddy et al., 2006; Mayr et al., 2009). In the current study, discordant results between CISH and local IHC testing as well as between CISH and central IHC testing were 30% and 6.3%, respectively. The comparison between the results of this study and the results from other studies show that the accuracy of IHC tests performed in the local laboratories due to following factors is not met criteria of ASCO/CAP guidelines for HER2 assessment:

1. As the majority of local laboratories used different ASCO/CAP guidelines (cut off point 10% or 30% for HER2 positivity) to interpret HER2 IHC tests, there was high rate of false- positive cases (discordance result among 3+IHC was 40%). This can explain the significant difference between local and central IHC results. The removal of cases scored as 2+ in IHC showed no significant differences between local and central IHC (ASCO/CAP new guidelines were used in central laboratory).

2. ASCO/CAP guidelines have been developed based on the comparison between IHC and FISH. Although there is high concordance between CISH and FISH and CISH

is an alternative to FISH, the differences in the results can be attributed to whatever mentioned above.

3. Using non-validated HER2 kits or antibodies. Although insufficient data is available, it seems that the type of antibody used in various laboratories to perform IHC staining can affect the accuracy of the test. Using FDA-approved kits such as Dako Hercept test can overcome this problem.

4. Preanalytical factors especially duration and type of tissue fixation influence HER2 receptor status by IHC/CISH tests. These preanalytical variables which were uncontrollable in this study were regarded as major factors influenced the IHC accuracy.

5. The last but not the least factor is absence of comprehensive external and internal quality control programs in local laboratories. This can directly affect Her2 results.

The comparison between sensitivity (90%) and specificity (57.7%) of local IHC with central IHC show that there is a high rate of false-positive HER2 in the local laboratories which not only causes unnecessary catastrophic overtreatment but also waste a lot of money. Such problems may lead to CISH or FISH test as the first step in HER2 assessment. Farshid et al in the article published in 2010 in Australia suggested one of the ISH methods (CISH or FISH) as the first step for HER2 assessment (Farshid et al., 2010).

Our results revealed that false positive HER2 test in our country is increased; however ministry of health is now trying to urge laboratories to follow ASCO/CAP guidelines. Regarding to importance of inaccurate HER2 testing led to inappropriate costly treatment and adverse unwanted drug toxicity, new guidelines of 2014 ASCO/CAP have to be strictly taken into account for every laboratories are intended to do IHC test for HER2. The concordance between IHC tests performed at various centers and gold standard CISH/FISH tests should be taken into consideration as well.

## Acknowledgements

## References

Ariga R, Zarif A, Korasick J, et al (2005). Correlation of her-2/neu gene amplification with other prognostic and predictive factors in female breast carcinoma. *Breast J*, **11**, 278-80.

Arnould L, Denoux Y, MacGrogan G, et al (2003). Agreement between chromogenic in situ hybridisation (CISH) and FISH in the determination of HER2 status in breast cancer. *British J Cancer*, **88**, 1587-91.

Bhargava R, Lal P, Chen B (2005). Chromogenic in situ hybridization for the detection of her-2/neu gene amplification in breast cancer with an emphasis on tumors with borderline and low-level amplification does it measure up to fluorescence in situ hybridization? *American J Clinical*

*Pathol*, **123**, 237-43.

Bilancia D, Rosati G, Dinota A, et al (2007). Lapatinib in breast cancer. *Ann Oncol*, **18**, 26-30.

Chang E, Lee A, Lee E, et al (2004). HER-2/neu oncogene amplification by chromogenic in situ hybridization in 130 breast cancers using tissue microarray and clinical follow-up studies. *J Korean Med Sci*, **19**, 390-6.

Dandachi N, Dietze O, Hauser-Kronberger C (2002). Chromogenic in situ hybridization: a novel approach to a practical and sensitive method for the detection of HER2 oncogene in archival human breast carcinoma. *Laboratory Investigat*, **82**, 1007-14.

Farshid G, Armes JE, Bell R, et al (2010). Establishment of the Australian in situ hybridization program for the assessment of HER2 amplification in breast cancer: a model for the introduction of new biomarkers into clinical practice. *Diagnostic Molecular Pathol*, **19**, 187-93.

Ferrero-Poüs M, Hacène K, Bouchet C, et al (2000). Relationship between c-erbB-2 and other tumor characteristics in breast cancer prognosis. *Clinical Cancer Res*, **6**, 4745-54.

Gupta D, Middleton LP, Whitaker MJ, et al (2003). Comparison of fluorescence and chromogenic in situ hybridization for detection of HER-2/neu oncogene in breast cancer. *Am J Clin Pathol*, **119**, 381-7.

Huang H, Neven P, Drijkoningen M, et al (2005). Association between tumour characteristics and HER-2/neu by immunohistochemistry in 1362 women with primary operable breast cancer. *J Clin Pathol*, **58**, 611-6.

Ivkovic-Kapicl T, Knezevic-Usaj S, Panjkovic M, et al (2007). HER-2/neu overexpression in invasive ductal breast cancer: An association with other prognostic and predictive factors. *Arch Oncol*, **15**, 15-8.

Kakar S, Puangsuvan N, Stevens JM, et al (2000). HER-2/neu assessment in breast cancer by immunohistochemistry and fluorescence in situ hybridization: comparison of results and correlation with survival. *Molecular Diagnosis*, **5**, 199-207.

Kumamoto H, Sasano H, Taniguchi T, et al (2001). Chromogenic in situ hybridization analysis of HER-2/neu status in breast carcinoma: Application in screening of patients for trastuzumab (Herceptin®) therapy. *Pathol International*, **51**, 579-84.

Laakso M, Tanner M, Isola J (2006). Dual-colour chromogenic in situ hybridization for testing of HER-2 oncogene amplification in archival breast tumours. *J Pathol*, **210**, 3-9.

Lan C, Liu JM, Liu TW, et al (2005). erb-b2 amplification by fluorescence in situ hybridization in breast cancer specimens read as 2+ in immunohistochemical analysis. *Am J Clin Pathol*, **124**, 97-102.

Madrid MA, Lo RW (2004). Chromogenic in situ hybridization (CISH): a novel alternative in screening archival breast cancer tissue samples for HER-2/neu status. *Breast Cancer Res*, **6**, 593-600.

Mayr D, Heim S, Weyrauch K, et al (2009). Chromogenic in situ hybridization for Her-2/neu-oncogene in breast cancer: comparison of a new dual-colour chromogenic in situ hybridization with immunohistochemistry and fluorescence in situ hybridization. *Histopathol*, **55**, 716-23.

O'Malley FP, Thomson T, Julian J, et al (2008). HER2 testing in a population-based study of patients with metastatic breast cancer treated with trastuzumab. *Arch Pathol Lab Med*, **132**, 61-5.

Park K, Kim J, Lim S, et al (2003). Comparing fluorescence in situ hybridization and chromogenic in situ hybridization methods to determine the HER2/neu status in primary breast carcinoma using tissue microarray. *Modern Pathol*, **16**, 937-43.

Piccart M, Mano M, Lohrisch C, et al (2002). Herceptin for the

treatment of breast cancer: what we know-and what we have yet to learn. *Cancer Futures*, **1**.

Reddy JC, Reimann JD, Anderson SM, et al (2006). Concordance between central and local laboratory HER2 testing from a community-based clinical study. *Clinical Breast Cancer*, **7**, 153-7.

Slamon DJ, Leyland-Jones B, Shak S, et al (2001). Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *New England J Med*, **344**, 783-92.

Tanner M, Gancberg D, Di Leo A, et al (2000). Chromogenic in situ hybridization: a practical alternative for fluorescence in situ hybridization to detect HER-2/neu oncogene amplification in archival breast cancer samples. *Am J pathol*, **157**, 1467-72.

Thomson TA, Hayes MM, Spinelli JJ, et al (2001). HER-2/neu in breast cancer: interobserver variability and performance of immunohistochemistry with 4 antibodies compared with fluorescent in situ hybridization. *Modern Pathol*, **14**, 1079-86.

Vera-Roman JM, Rubio-Martinez LA (2004). Comparative assays for the HER-2/neu oncogene status in breast cancer. *Arch Pathol Lab Med*, **128**, 627-33.

Vogel UF (2010). Confirmation of a low HER2 positivity rate of breast carcinomas-limitations of immunohistochemistry and in situ hybridization. *Diagn Pathol*, **5**, 16.

Wolff AC, Hammond MEH, Hicks DG, et al (2013). Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: american society of clinical oncology/college of american pathologists clinical practice guideline update. *J Clin Oncol*, **31**, 3997-4013.

Wolff AC, Hammond MEH, Schwartz JN, et al (2006). American society of clinical oncology/college of american pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *J Clin Oncol*, **25**, 118-45.

Yarden Y, Baselga J, Miles D (2004). Molecular approach to breast cancer treatment. seminars in oncology, *Elsevier*, 6-13.

Zhao J, Wu R, Au A, et al (2002). Determination of HER2 gene amplification by chromogenic in situ hybridization (CISH) in archival breast carcinoma. *Modern Pathol*, **15**, 657-65.

100.0

75.0

50.0

25.0

0

6.3

56.3

31.3

**Newly diagnosed without treatment**