

Determination of HER2 Gene Amplification using Chromogenic in Situ Hybridization (CISH) in Iraqi Patients with Breast Carcinoma

Basim Mohammed Khashman¹, Safana Abdul-Sattar²

¹M.Sc, Iraqi National Cancer Research Center (NCRC), University of Baghdad

²M.Sc, Iraqi National Cancer Research Center (NCRC), University of Baghdad

Abstract: ***Background:** Breast cancer is the most common malignancy among women worldwide. The human epidermal growth factor receptor (HER) is a transmembrane receptor in the epidermal growth factor (EGF) receptor family located at the long arm of human chromosome 17 (17q12). Diagnostic assays for HER2 in breast cancer provide important prognostic information and independently help guide management by identifying patients who are the most likely to benefit from Herceptin-targeted therapy. **Objective:** To evaluate the Efficiency of using CISH technique in Equivocal Immunohistochemical results of HER2 protein. **Materials and Methods:** Sixty cases of breast cancer were subjected to immunohistochemistry for expression of HER2 protein. The genetic status of HER2 gene were determined using Chromogenic In Situ Hybridization (CISH) to discriminate the equivocal results of score 2 immunohistochemical expression. **Results:** within the Equivocal cases, 24.5 % (12 out of 49) showed low amplification results while 8.1% (4 out of 49) showed high amplification results the rest showed no amplification result. **Conclusion:** Integration of CISH in Iraqi diagnostic laboratories during HER2 testing algorithm of all equivocal HER2 IHC(2+) test result provides a convenient definitive guidance for accurate HER2 gene amplification status to discriminate these results.*

Keywords: Breast Cancer, Chromogenic in Situ Hybridization, human epidermal growth factor receptor

1. Introduction

Breast cancer is the most common malignancy among women worldwide (Basim, 2013). In Iraqi populations, breast cancer constitutes about one fourth of the registered cancer cases (Nada et al 2014).

There are several well-established risk factors for breast cancer (early onset of menarche, a late age both for a first complete pregnancy and for menopause, the presence of atypical hyperplasia, a positive family history of breast cancer, and exposure to ionizing radiation).. (Hussain et al, 2016). Globally and According to GLOBOCAN, the prevalence of Breast cancer is 1,461,445 worldwide with the incidence of 1,824,701 and mortality of 1,589,925 (<http://gco.iarc.fr/today/>).

The human epidermal growth factor receptor (HER) is a transmembrane receptor in the epidermal growth factor (EGF) receptor family located at the long arm of human chromosome 17 (17q12) (Coussens et al; 1985). HER belongs to family of receptors plays a central role in the pathogenesis of several human cancers. They regulate cell growth, survival, and differentiation via multiple signal transduction pathways and participate in cellular proliferation and differentiation. (Nida et al; 2014.)

Diagnostic assays for HER2 in breast cancer provide important prognostic information and independently help guide management by identifying patients who are the most likely to benefit from Herceptin-targeted therapy. Immunohistochemistry and ISH methodologies have the advantage of being morphologically driven, allowing for correlations between HER2 expression and morphologic features. However, each has important advantages and disadvantages. Although immunohistochemistry is familiar

and readily accommodated in most surgical pathology laboratories, increasing demands for ISH testing in the clinical setting will require greater familiarity with the technical aspects of ISH assays and their interpretation by the greater laboratory community. In this review, we provide an overview of CISH testing for HER2 in breast cancer (David et al; 2005.)

2. Objective

To evaluate the Efficiency of using CISH technique in Equivocal Immunohistochemical results of HER2 protein.

3. Materials and Methods

This study is retrospectively designed in which 60 cases which were diagnosed having breast cancer in the National Cancer Research Center and Central Public Health Laboratory during the period from October /2015 till June /2015 were evaluated in terms of age, tumor type and grade.

Breast tissue sections were cut at 4 µm and placed on positively charged slides; one section was stained with hematoxylin and eosin (H&E) and the others were subjected for the Immunohistochemistry technique (IHC) to detect the expression of ERB2 which were done by the Central Public Health Laboratory, Iraqi Ministry of Health. Chromogenic In Situ Hybridization (CISH) was performed using the ZytoDot 2C SPEC HER2/CEN 17 Probe Kit, (Zytovision, Germany) according to the manufacturer's instructions. The kit was designed to be used for the detection of the human HER2 gene as well as chromosome 17 alpha-satellites in either formalin-fixed, paraffin-embedded tissue or cell samples by chromogenic in situ hybridization (CISH).

4. Interpretation of Results

The interpretation was followed the manufacturer instructions of ZytoDot 2C SPEC HER2/CEN 17 Probe Kit (figure 1). The signal of single copy of a HER2 gene appeared as a dark green-colored distinct dot-shaped signal, while the bright red-colored distinct dot-shaped signal indicate one single copy of a chromosome 17 centromeric region. For distinguishing the colors contrast, we used hematoxylin as a counterstain. In normal diploid nuclei without gene amplification, 2 green and 2 red dotshaped signals with smooth, rounded edges will be visible per nucleus. Due to mitosis, additional signals may be visible even in a small percentage of non-neoplastic cells. Occasionally, nuclei with missing signals may be observed in paraffin-embedded tissue sections. In case of low gene amplifications or chromosome 17 aneusomy, green HER2 gene specific signals will be visible as multiple dots or small clusters. Small clusters are irregularly shaped signals comprising an area of up to 5 dots. As a reference, a single green dot of a normal cell of the same slide must be used. Additionally, red signals of the centromere 17 control will be visible. In case of high gene amplifications, a large number of green dots or large clusters, comprising an area greater than 5 dots, will be visible in the nuclei. As a reference, a single green dot of a normal cell of the same slide must be used. Red signals might be overlaid and might not be visible any more. (Zytovision, Germany).

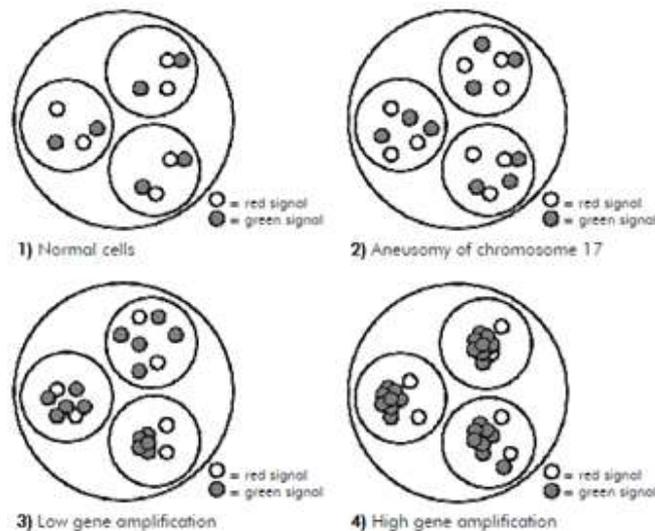


Figure 1: Interpretation of the CISH results (source: www.Zytovision.com)

Statistical analysis

Data was analyzed using statistical package of social sciences (SPSS) for version 10, Social Science Statistic (<http://www.socscistatistics.com>) and office Excel 2010. Differences in proportions were assessed by Chi-square test. P-values < 0.05 were considered statistically significant

5. Results

This research was designed as a retrospective study. A total of 60 cases Formalin fixed paraffin embedded archival tissues from Iraqi women with breast cancer were included in this research. The minimum age was 25 years and maximum was 65 years with the most affected age group 45-54 years. According to the type of cancer, the most prominent type is the ductal carcinoma (38 out of 60) while the lobular carcinoma comprises only 8% of the total cases (5 out of 60) (Figure 2). There is 17 cases of unknown origin of cancer. Most of the cases were at stage 3 Grade 2 which represent 20% and 65% of the total cases respectively (Figure 2). Because of the missing data in the case sheets during the collection the sample, we will focus on the expression of both HER2 gene and protein.

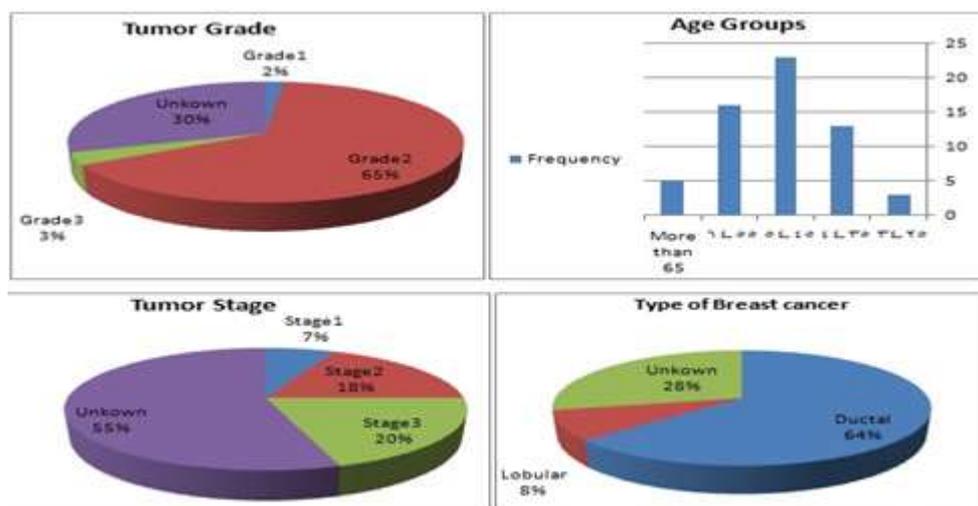


Figure 2: Sample description

The immunohistochemical expression of HER2 protein showed that the majority of the cases were at score2 (49 out of 60) followed by score1 (10 out of 60) , only one case showed score3 expression for HER2.(table 1).

Table 1: Immunohistochemical expression of HER2 protein

HER2	Frequency	Percent	significance
SCORE1	10	16.7	0.037 significant
SCORE2	49	81.7	
SCORE3	1	1.6	
Total	60	100	

Detection of Her2 gene amplification showed graded amplification result from no-amplified gene followed by low-amplification then high-amplified result with a percentage of 71.7%, 21.7% and 6.7% for each respectively, there is a significant correlation of CISH test in the detection of gene amplification in breast cancer tissues. (Table 2).

Table 2: Gene expression of the HER2 using CISH

CISH	Frequency	Percent	Significance
no-amplified	43	71.7	0.026 significant
low-amplified	13	21.7	
high-amplified	4	6.6	
Total	60	100	

Among the 49 cases of equivocal score2 expression, which is the most interesting expression that need to be confirmed by detection of Her2 gene, only 4 cases showed highly-amplification result (Table 3) with a significant correlation between protein expression and gene amplification while 12 cases were low amplified her2 gene with statistically non significant correlation (p-value 0.275).

Table 3: The correlation between Immunohistochemistry expression and the results of CISH gene amplification

CISH Amplification	Equivocal Score2 IHC expression	Percentage	Chi-square
no-amplified	33	67.4	2.577 P=0.275 Non-significant
low-amplified	12	24.5	
high-amplified	4	8.1	
Total	49	100%	

6. Discussion

Among new breast cancer patients, 15% to 20% will develop tumors that harbor a genomic alteration involving the HER2 gene locus. This alteration results in amplification of the region on chromosome 17 containing this proto-oncogene.(David and Linda ,2011)

HER2 gene amplification is associated with shorter disease-free and overall survival in breast cancer. (D. J. Slamon et al , 1987). HER2 is a clinically important tumor marker with the potential to influence treatment decisions in the breast cancer patient, a role among tumor markers that previously has been achieved only with hormone receptors.

Abnormal HER2 (gene amplification or protein overexpression) is also important in selecting metastatic breast cancer patients who are candidates for chemotherapy. (Lynn et al, 2005)

Despite The IHC has the advantage of being morphologically driven, allowing for correlations between HER2 expression and morphologic features in tissue sections (David et al 2005.) which is widely accessible and easy to perform at a reasonable cost , this semi-quantitative procedure is beset by technical artifacts, sensitivity differences between different antibodies, and subjective interpretation, resulting in interobserver variability between pathologists. This is obvious when we compare the results of table 1 and table 3 in which although there is statistically non significant correlation in table 3 ,we found that the 67% of the equivocal results detected by IHC had non-amplified HER2 gene when subjected to CISH, this explained the importance of CISH application during the use of IHC in the detection of HER2 protein over expression (Table 2) (Press et al,1994)

Kakar and colleagues concluded that IHC is appropriate for the initial Her-2/neu assessment, but patients whose tumors scored less than 3+ (particularly those interpreted as 2+) would benefit from genetic detection by In situ hybridization to assess Her-2/neu status more accurately and to avoid inaccurate prognostication and inappropriate treatment and this is support our findings in table 2 and 3 . (Manuelito and Raymundo 2004) During this study we found that the despite the use of IHC approach is useful for the initial detection for Her-2 protein expression , the patients with equivocal score 2 IHC need to use more accurate methods including the evaluation of gene amplification .like cish or fish to avoid inaccurate prognostication and inappropriate treatment and this finding is in agreement with Kakar and colleagues. (Kakar et al,2000) and that is because the difference in the target ; Immunohistochemistry identifies HER2 protein expression on the cell surface, while CISH determines the actual degree of HER2 gene amplification (van de Vijver,2002).

Chromogenic in-situ hybridization considered as a viable alternative to fluorescence in-situ hybridization in the HER2 testing algorithm Many studies find that using CISH is more accurate than FISH besides unlike FISH, positive signals can be identified using standard laboratory equipment. Histopathology of the specimen can be assessed simultaneously and the signal does not decay, but remains stable over a long period of time, allowing slides to be stored at room temperature .(Wedad and Kevin 2006) and that is why we used CISH in this study.

The correlation of IHC with CISH was crucial to determine which of the IHC score 2 equivocal cases were eligible for chemotherapy (Marc et al,2007). In this study about 32.6% of the 49 equivocal cases of HER2 showed HER2 gene amplification as assayed by CISH (table3). This is in consistent with the findings of other studies comparing HER2 over expression with the degree of HER2 gene amplification. In these studies, the percentage of tumours with a HER2 IHC 2+ score and gene amplification was approximately 25% (Lebeau et al; 2001, Mass et al;2000, Tsuda et al;2001). Moreover other researchers found that HER2 is over expressed in 25–30% of all breast cancers (Cobleigh et al, 1999; Andersson et al, 2002) .

Despite The advantage of being use CISH in excluding equivocal results, we thought that the pre-treatment of tissue sections, especially the pepsin digestion time, was a critical step in achieving a good CISH result. Like FISH, the optimal pepsin digestion time differs between tumours. For practical reasons, the pepsin digestion time used in the preparation of received slides was calculated in each laboratory on the basis of a pepsin time course performed on one representative tumour provided by the sending laboratory. However, it is possible that this was not the optimal value for all tumour samples, and as a result it was likely that in some cases no HER2 signal would be detected. For such cases, it was determined that the CISH test should be repeated after adjustment of the pepsin digestion time. This may explain the different results of CISH between the researchers. (Marc et al, 2007).

In conclusion , this study suggest that it is important to standardize tissue handling, fixation, use of standardized assays, rigorous quantitation, quality control measures, and competency assessments are a prerequisite to this approach and will help ensure accuracy and consistency for IHC HER2 evaluation . Moreover; we recommend to integrate CISH in Iraqi diagnostic laboratories than FISH during HER2 testing algorithm of all equivocal HER2 IHC(2+) test result which provides a convenient definitive guidance for accurate HER2 gene amplification status with a minimum requirements of equipments which were available in most of Iraqi pathological diagnostic laboratories.

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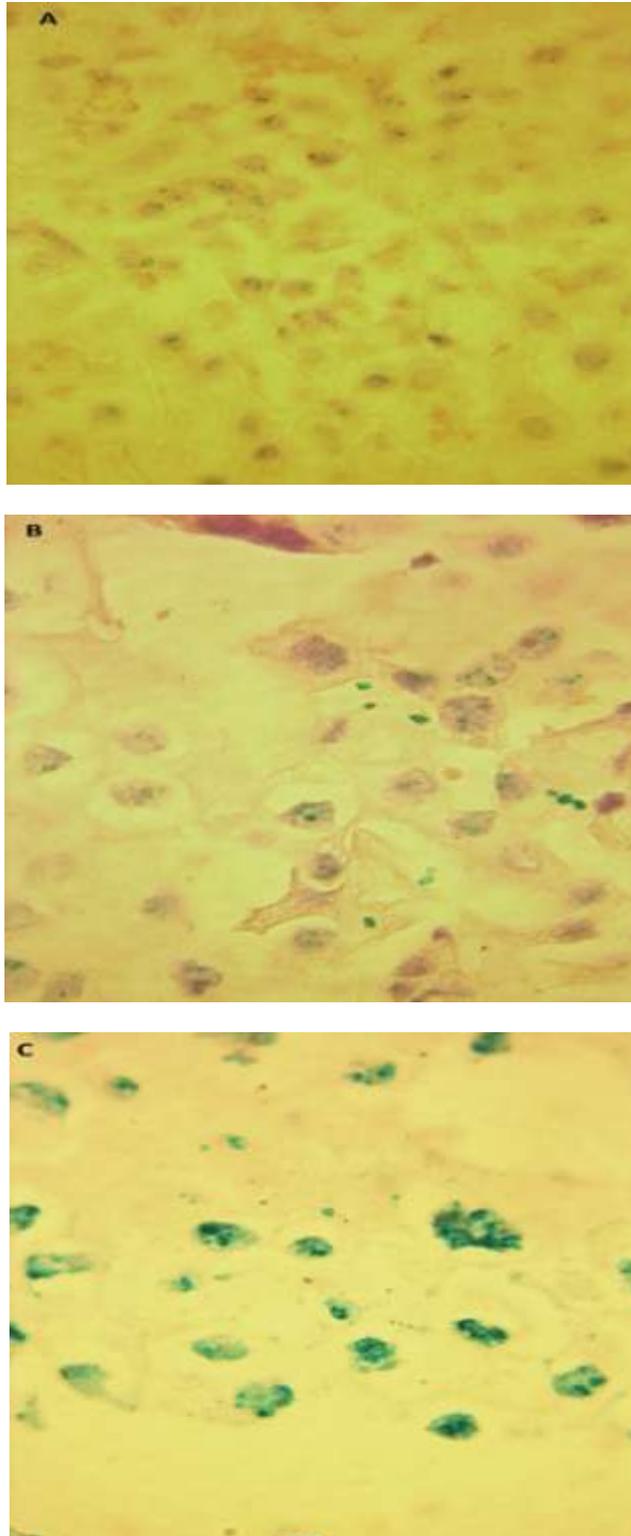


Figure 3: Genetic expression results of HER2/neu using Chromogenic in situ hybridization CISH: (A) Non amplification. (B) Low amplification. (C) High amplification