

# Dual Color -Chromogenic *in Situ* Hybridization Approaches to Evaluate HER2/Neu Gene Amplification in Breast Carcinomas

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## ABSTRACT

Breast cancer continue to be the most frequent malignant tumor in women, constituting the second most common fatal malignant tumor in women. The validated prognostic HER2/neu. Aim of the study:- Evaluating HER-2 by immunohistochemical (IHC) for protein expression and *in situ* hybridization (ISH) by dual-color chromogenic *in situ* hybridization (DC-CISH) technique for HER-2 gene copy amplification and chromosome 17/CEN (chr.17 CEN) aneuploidy in breast cancer.

**Methods:** This is a prospective study, where by fifty-pairs of fresh tissues from both breast cancer of invasive ductal carcinoma and apparently normal adjacent tissues (NATs) which considered as healthy normal internal control. we select only positive cases of HER-2 /neu by IHC (16 cases which included score+2 and score +3 HER-2 by IHC) for gene amplification by DC-CISH were done in CPHL/Baghdad.

**Results:** Out of 6 cases with her2(score +2) in breast cancer by IHC expression, 2 (33.33%) show no amplification, while all cases4(66.67%), show low amplification. All cases with (score +3) by IHC, showed gene amplification. Out of 10 cases with (score +3) by IHC, 2 cases (20%) showed low amplification while majority of cases,8 (80%) showed high amplification.

**Conclusion:** The DC-CISH is confirmatory technique for the detection of both HER-2 gene amplification and multiplication of chromosome 17/CEN in breast cancer.

**Keywords:** Breast cancer, Her/2neu, IHC, DC-CISH

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## INTRODUCTION

Breast cancer continue to be the most frequent malignant tumor in women, constituting the second most common fatal malignant tumor in women (1). In Iraq, in the incidence rate of breast carcinoma, making it a common horrible cause of women mortality and morbidity (2). A corner stone modality in fighting breast cancer, during last decades, has been directed of research works toward understanding the expression and function of major signaling pathways in carcinoma initiation and subsequent progression (3). These strategies allowed the uncovering of breast cancer subsets with variable biologic behavior. Recently, several prognostic and predictive factors for breast cancer lie estrogen, progesterone and human epidermal growth factor receptors have been evaluated. Human epidermal growth factor receptor is a representative of the human epidermal growth factor receptor (human EGFR) family; it is a transmembrane growth factor receptor that has a tyrosine kinase activity. Human epidermal growth factor receptor 2 (HER-2) is situated on chromosome 17q21 also referred to as neu or c-erbB-2 (4).

The HER-2 proto-oncogene has been evaluated in breast cancer patients to identify those most likely to benefit from herceptin-targeted therapy. HER2 amplification, detected in 20-30% of invasive breast tumors, is associated with reduced survival and metastasis. The most frequently used technique for evaluating HER2 protein status as a routine procedure is immunohistochemistry (IHC). HER-2 copy number alterations have also been evaluated by fluorescence *in situ* hybridization (FISH) in moderate immunoexpression (IHC 2+) cases. An alternative procedure to evaluate gene

amplification is chromogenic *in situ* hybridization (CISH), which has some advantages over FISH, including the correlation between HER2 status and morphological features. Other methodologies have also been used, such as silver-enhanced *in situ* hybridization (SISH) and quantitative real-time RT-PCR, to determine the number of HER2 gene copies and expression, respectively. Here we will present a short and comprehensive review of the current advances concerning HER2 evaluation in human breast cancer (5).

## PATIENTS MATERIAL AND METHODS

The study was conducted during the period from January 2013 to January 2015. This is a prospective study, whereby patients were recruited at the Surgical Department/ AL-Diawania Teaching Hospital in Diawania city. Fifty patients diagnosed as having breast cancer who were subjected to the three principle evaluation methods: physical examination, imaging techniques (mammography and /or ultrasound) and (FNAC). Patients were analyzed for clinical data with special focusing on the age. Their ages range (30 -76) years with mean was (47+1.71) years, all cases underwent modify radical mastectomy and axillary clearance and no preoperative adjuvant chemotherapy or target therapy. Fifty-pairs of fresh tissues from both breast cancer of invasive ductal carcinoma and apparently normal adjacent tissues (NATs) which considered as healthy normal internal control. Marker assay and then we select only positive cases of HER-2 /neu by IHC (16 cases which included score+2 and score +3 HER-2 by IHC) for gene amplification by DC-CISH were done in CPHL/Baghdad .

**Processing and staining**

After 48h fixation of representative pieces in 10% of formalin, these pieces were processed in graded alcohol, xylene and paraffin as manual tissue processing. After that blocks were made (each paraffin embedded block containing pair tissues (tumor and NATs) for every case, then sectioned at 4 micrometer thickness then subjected to H&E conventional stain.

**Immunohistochemistry (IHC).**

**Principle of the test.** It is to localize antigens in tissue sections by use labeled antibodies as specific reagents and through antigen -antibody interactions that are visualized by a reporter molecule such as fluorescent dye, enzyme, radioactive element.

**Preparation of tissue sections**

Tissue sections of about four to five micrometer thickness were adhered into positively enhanced slides in order to evaluate HER-2 status IHC. NATs (considered as internal control).

**(A)-Positive Control sections:** concomitant positive control tissue specimens have been run with each IHC procedure. These tissues were obtained from breast tumors already proved to be positive for HER-2.

**(B)- Negative Control sections:** these were obtained by technically omitting the primary antibody from the routine IHC procedure.

**Immunohistochemical staining procedure**

This was according to the protocol Streptavidin Biotin (LSAB+) as shown in kit (6).

**Staining results**

brown membranous stain was the positive yield for HER-2.

The depth of reaction was evaluated according to that recommended by Sophia et al.( 1999) , through estimation of the number of reactive cells in (100 cancer cells) at objective (40X) total magnifications, counting include five high power field per sample (7).

**Quality of staining:** Any detectable staining by low power (4X) was considered strong, while any color that was detected by mean of high power (40X) only was considered weak pattern.

**Scoring system for HER-2/neu:**

**Quality evaluation:**

The same principle mentioned above for ER and PR, but the reaction here is membranous rather than nuclear (8). As shown in table (1)

**Table 1:** IHC staining score concerning HER-2/neu

Staining pattern	Score	HER-2
A strong complete membrane staining is observed in more than 30% (formerly 10%) of the tumor cells	3+	Strongly positive
A weak to moderate complete membrane staining is observed in more than 10% of the tumor cells	2+	Weakly positive (equivocal)need ISH
A faint/barely perceptible membrane staining is detected in more than 10% of the tumor cells. The cells are only stained in part of their membrane	1+	Negative
No staining is observed, or membrane staining is observed in less than 10% of the tumor cells	0	Negative

**Dual-Color Chromogenic Insitu Hybridization.**

**Principle of the test:** A polynucleotides probe labeled with digoxigenin in will attack HER-2 genome sequences, and a polynucleotides probe labeled with Dinitrophenol (DNP) which attack chromosome 17/CEN.

A primary antibody is used to visualize the labeled probe duplex complex, then this would be detected by secondary antibody, which is polymerized, and enzyme attached. The chemical reaction will lead to stable intense green and red marks that can be demonstrated by use of conventional microscope at (40X) dry lens (5).

**Preparation of tissue sections**

Sixteen specimens of representative paraffin embedded blokes which contained (both IDC and NATs) for HER-2 positive by IHC, score (+2, n=6) which considered as equivocal cases, and score (+3, n=10) . All 16 specimens mentioned above were utilized to get sections of 4 μm thickness attached to positively charged slides utilized to evaluate the HER-2 gene copy / 17CEN ratio by using new DC- CISH technology of Zytovision and the procedure were done in CPHL/Baghdad . This protocol permits sophisticated specificity and little false positive stain because of specific Zytovision Repeat Subtraction procedure and is typified by great sensitivity owing to enzyme -coupled polymers for the identification of HER-2 gene augmentation.

**(A)Positive control:** A case of breast carcinoma, with (score 3+) IHC HER-2 protein expression, showing gene amplification by DC-CISH was used as a positive control.

**(B)Negative control:** Serial tumor sections in which TBS was used instead of HER-2 probe were used as negative controls.

**Staining Protocol for HER-2/neu by DC-CISH**

This protocol done according to company instructions leaflet as following steps (9):

**Scoring system for HER-2/neu and interpretation of results by DC-CISH**

The CISH hybridization signal of one single copy of a HER-2 gene appears as a dark green-colored distinct dot-shaped signal, the signal of one single copy of a chr.17/ CEN region appears as bright red-colored distinct dot-shaped signal, which can be clearly distinguished from the background counter stained with hematoxylin . Visualization of signals should be performed under light microscopy at power (40X) whereas the counting of positive cells was performed at oil emersion (X100). Resulting in easily visible signals. Be sure to focus up and down when evaluating a nucleus as red and green signals might be located on top of each other

Prior to signal enumeration, the tissue should be scanned for any possible intra-tumoral heterogeneity using a (10X or 20X) objective. In case of heterogeneity, an area representative for the amplification status has to be chosen. For signal enumeration, area of necrosis, overlapping nuclei, and nuclei with weak signal intensity should be avoided, only nuclei with a distinct nuclear border of invasive tumor cells being evaluated.

In normal diploid nuclei without gene amplification, 2 green and 2 red dot-shaped 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 cell. Occasionally, nuclei with missing signals may be observed in paraffin-embedded tissue sections.

Hybridization signals were counted in 50 nuclei per sample. The interpretation followed the criteria of Pauletti and coworkers (10) in accordance with the new recommendations of the American Society Cancer Oncology/College of American Pathologists (ASCO / CAP) (11) : negativity for HER-2 gene amplification when ISH ratio is <1.8; positivity for HER-2 gene amplification when ISH ratio is >2.2. Cases with ISH ratio 1.8-2.2 consider as equivocal. Subsequently, after counting 100 nuclei per sample, or after repeating the ISH were evaluated as equivocal, the cutoff ratio of ISH for HER-2/17CEN, equal to and more than 2( $\geq 2$ ) , were conceded as amplified.

An average count of chr. 17/CEN ( $\geq 2.6$ ) per nucleus was considered as polysomy while when average count of chr.17/CEN (<2.6) per nucleus was considered as normal (either monosomy or disomy) and no polysomy (12). Concerning to HER-2 gene amplification ,the assessment of HER-2 status, not only as a ratio of HER-2/ chr.17CEN but also absolute HER-2 gene copy numbers and absolute chr.17/CEN number (use chr.17/CEN as reference genes might more accurately assess true HER-2 gene status)

should be analyzed, in order to minimize false-positive and negative test results , because if chr.17/CEN is co-amplified along with the HER-2 gene (i.e. , as part of the same amplicon),chr.17/CEN might not be an appropriate reference gene for the status of chr. 17/CEN, and the calculated HER2: 17/CEN ratio may mask the presence of true amplification of the HER2 gene. In case of low gene amplification or chr.17 aneusomy, HER-2 gene specific signals will be visible as multiple dots or small clusters .In case of high gene amplification ,a large number of green dots or large clusters ,comprising an area greater than 5 dots ,will be visible in the nuclei .

**Statistical analysis:** -SPSS version 16 and Microsoft Office Excel 2007 were using in analysis of these data; Chi-square test and Fisher exact test were used to study association between any two nominal variables. P-value of less than or equal to 0.05 was considered significant.

**RESULTS**

**Human epidermal growth factor receptor-2 protein expression**

Patients with positive IHC HER-2 expression accounted for 16 (32%) only. Patients with score +3 represented a large proportion, 10 (20%), while patients with score+2 accounted for 6 (12%) which consider as equivocal cases, as shown in table (2). The HER-2 IHC illustrated as figures (1), (2) and (3).

**Table 2:** IHC for HER-2 expression in patients with breast carcinoma

HER-2/neu score*	No.	%
0 (-ve)	32	64
1 (-ve)	2	4
2 (+ve)	6	12
3 (+ve)	10	20
Total	50	100

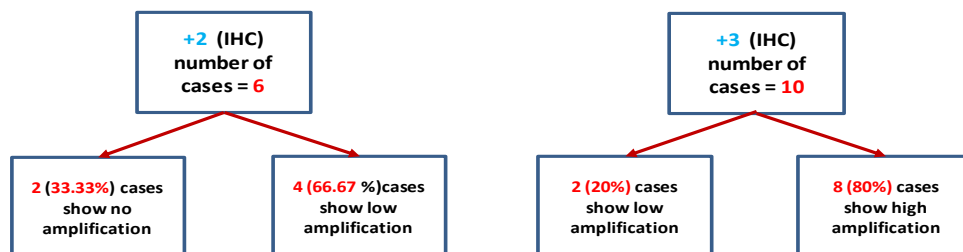
\*Patients with score 0 and score 1 were regarded as negative, while patients with score 2 and 3 were regarded as positive.

**Human Epidermal Growth Factor Receptor-2 gene amplification by Dual color–chromogenic insitu hybridization**

Out of 16 patients with positive HER-2 protein (score+2 and score +3 which enrolled in present study), gene amplification was done by DC-CISH.

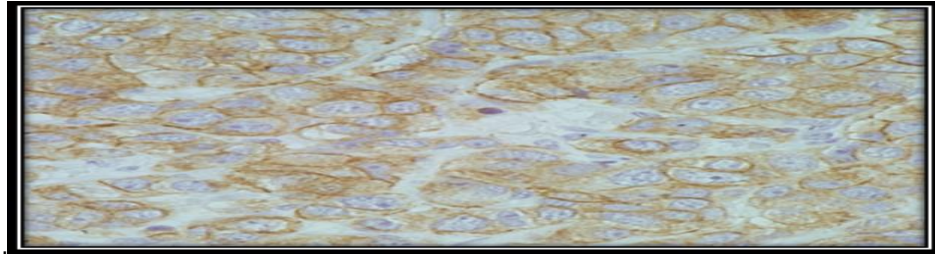
Out of 6 cases with (score +2) by IHC expression, 2 (33.33%) show no amplification, while all

cases4(66.67%), show low amplification. All cases with (score +3) by IHC, showed gene amplification. Out of 10 cases with (score +3) by IHC, 2 cases (20%) showed low amplification while majority of cases,8 (80%) showed high amplification, as shown in figure (4). DC-CISH for HER-2 and chr.17/CEN as shown in figures (5) and (6).

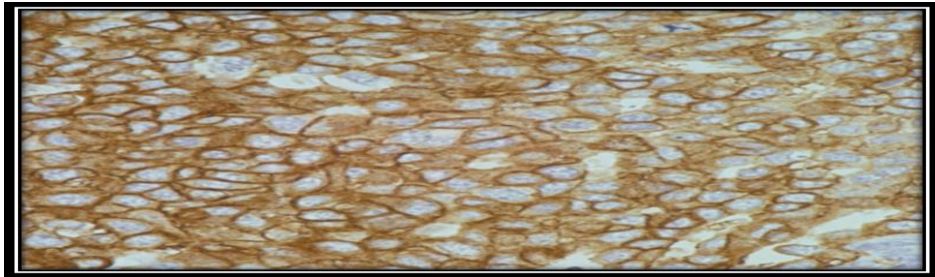


**Figure 4:** Relation between HER-2 IHC expression and DC-CISH result.

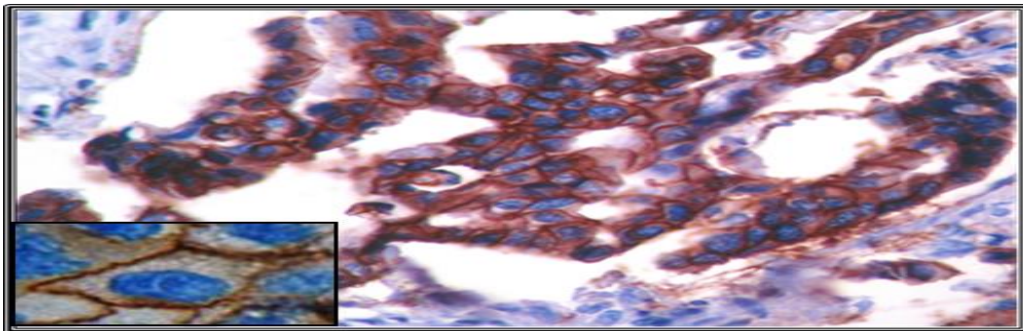




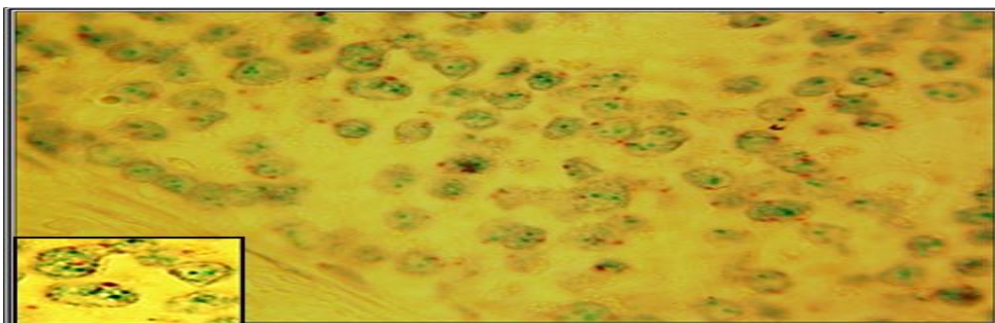
**Figure 1:** Breast, invasive ductal carcinoma. IHC for HER-2/neu, score +1. Arrow shown incomplete membrane staining with weak-moderate brown discoloration, more than 10% of tumor cell , (20X) .



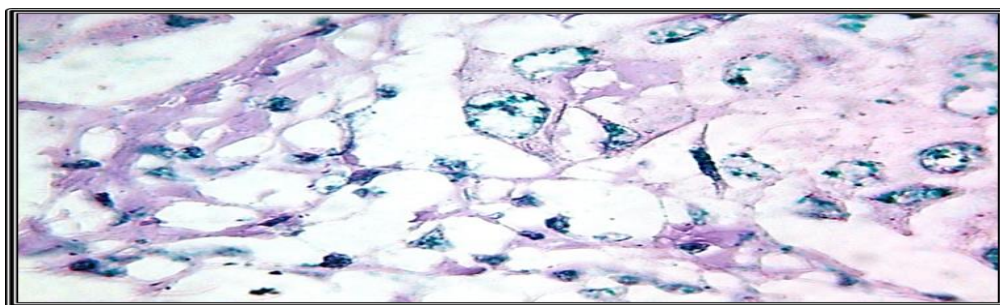
**Figure 2:** Breast, invasive ductal carcinoma. HER-2/neu positive score +2. Arrow shown complete membrane staining with weak - moderate brown discoloration was observed in more than 10% of the tumor cells, (20X).



**Figure 3:** Breast, invasive ductal carcinoma. HER-2/neu positive score +3. Arrow inside inset shown complete membrane staining with strong brown discoloration was observed in more than 30% (formerly 10%) of the tumor cells with fish net appearance, 20X.



**Figure 5:** Breast, invasive ductal carcinoma with normal HER-2 gene copy and normal chr.17/CEN by DC-CISH. Arrow inside inset shown (HER-2 gene appears as a dark green colored distinct dot-shaped signal while the signal of one single copy of a chr.17 CEN region appears as bright red-colored distinct dot- shaped signal) , 20X .



**Figure 6:** Breast, invasive ductal carcinoma with high amplification of HER-2 and normal chr.17/CEN by DC-CISH. Arrow (HER-2 gene appears as a dark, green-colored distinct dot-shaped signal while the signal of one single copy of a chr.17 CEN region appears as bright, red-colored distinct dot-shaped signal), (40X).

## DISCUSSION

Concerning to result of IHC for HER-2 protein, majority of cases were HER-2 negative ,68% and cases with HER-2 positive 32% only. These results were different with that results which conducted by (Gamber) <sup>(13)</sup>, in which majority of cases were positive HER-2, 63.2% while negative cases for HER-2 were ,36.8%. The differences could be due to the presented study was prospective study and (Gamber) <sup>(13)</sup> study was retrospective studies , while these results accepted with that result of those reported by (Hong *et al*) <sup>(14)</sup> whom found that the positive HER-2 were 26.7% .

### HER-2/neu gene amplification by DC-CISH

All 10 cases of IDC with positive HER-2 classified as +3 by IHC were amplified and majority of these cases, 8 (80%) showed high amplification .These findings are in agreement with others studies as reported by (Sophia *et al*) <sup>(15)</sup>; (Doris *et al*) <sup>(16)</sup> and (Beatriz *et al*) <sup>(17)</sup>, in which the cases of IDC with positive HER-2 classified as +3 by IHC were amplified by DC-CISH ,were ; 22(100%) ; 47(82.5%) and 11(100%), respectively and majority of them with high amplification . Regarding to result of IDC with positive HER-2 classified as score (+2) by IHC, the majority of cases , 4(66.67%) out of 6 cases ,were amplified and all of them with low amplified and only 2(33.33%) of cases with no amplification . These findings are accepted with others studies as reported by (Beatriz *et al*) <sup>(17)</sup>, in which , all cases 13(100%) of IDC with positive HER-2 classified as score (+2) by IHC, were amplified by DC-CISH and majority of these cases with low amplification. The results of present study were highly difference from those reported by (Sophia *et al*) <sup>(15)</sup> and (Doris *et al*)<sup>(16)</sup> , in which the proportion of IDC with positive HER-2 classified as score (+2) by IHC, were amplified by DC-CISH ,were 31% and 20.9% , respectively .

The cases with positive HER-2 by IHC and with no amplification by ISH, it may be caused by alternative transcriptional or post-transcriptional mechanisms controlling HER-2 expression, (Daohai *et al*) <sup>(18)</sup>. About to result of chr.17/CEN, knowing the level of polyploidy is essential for HER-2 status determination. DC-CISH results are easier to interpret if the number of chr.17CEN/tumor cell is known. Thus, the differential diagnosis of an actual gene amplification and HER-2 signal multiplication, which is due to chr. 17/CEN polyploidy is more easily established. Such heritable variation may carry a gloomy outcome in particular malignant breast tumor carrier (Sybren *et al*) <sup>(19)</sup>. All 16(100%) cases of present study were chr.17/CEN , with disomy which were normal and no any case with polysomy in present study , thus the

(14) cases with HER-2 amplification were the defect present in gene its self (which amplify) and no chromosome multiplication . These findings are in agreement with to those reported by (Doris *et al*) <sup>(16)</sup>; (Sybren *et al*) <sup>(19)</sup> and (Alina *et al*) <sup>(20)</sup>, in which the proportion of chr. 17/CEN disomy was 98.8%, 76.5% and 93.7%, respectively.

The findings of the current work contradict to the findings of (Downs *et al*) <sup>(21)</sup> and (Huiyong *et al*) <sup>(22)</sup>, who found that the proportion of chr. 17/CEN disomy was, (60%) and (53.1%), respectively.

Explanations for the disagreement between the present study and those other studies in correlation between IHC and DC-CISH, are differences in number of sample size, scoring system ,intra-tumor heterogeneity, type of antibody for HER-2(monoclonal or polyclonal), the result that positive detection by DC-CISH proved to be minimum when old paraffin blocks were examined as reported by (Jianxin *et al*) <sup>(23)</sup>.The most important point in present study were prospective study , early fixation and the duration of fixation not more than (48-72h) with good processing according to ASCO/CAP guide line .

## CONCLUSION

The DC-CISH technique is cost effective method to evaluate HER-2 gene amplification and multiplication of chr. 17/CEN in breast cancer.

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