Open Access Maced J Med Sci **electronic publication ahead of print,**
published on June 30, 2019 as https://doi.org/10.3889/oamjms.2019.342

ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences.
https://doi.org/10.3889/oamjms.2019.342
eISSN: 1857-9655
Basic Science

Chromogenic in Situ Hybridization Technique versus Immunohistochemistry in Assessment of HER2/neu Status in 448 Iraqi Patients with Invasive Breast Carcinoma

Ali Hussein Mohammed Ali[1], Alaa Qasim Yahya[2*], Haider Latteef Mohammed[3]

[1]Histopathology Unit, Central Public Health Laboratories, Baghdad, Iraq; [2]Department of Pathology, Al-Kindy College of Medicine, Baghdad, Iraq; [3]Department of Pathology, Al-Wasiti Teaching Hospital, Bagdad, Iraq

Citation: Mohammed Ali AH, Yahya AQ, Mohammed HL. Chromogenic in Situ Hybridization Technique versus Immunohistochemistry in Assessment of HER2/neu Status in 448 Iraqi Patients with Invasive Breast Carcinoma. Open Access Maced J Med Sci. https://doi.org/10.3889/oamjms.2019.342

Keywords: ERBB2; HER2/*neu*; Immunohistochemistry; Chromogenic in situ hybridisation; Breast carcinoma

***Correspondence:** Alaa Qasim Yahya. Department of Pathology, Al-Kindy College of Medicine, Baghdad, Iraq. E-mail: alaakasim1983@yahoo.com

Received: 07-Apr-2019; **Revised:** 22-May-2019; **Accepted:** 23-May-2019; **Online first:** 30-Jun-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abbreviation: IHC: Immunohistochemistry; FISH: Fluorescence in situ hybridisation; HER2: Human epidermal growth factor receptor 2; ASCO: American society of clinical oncology; FDA: Food and drug administration; CEN: centromere

Abstract

BACKGROUND: The rapidly growing knowledge regarding factors controlling tumour growth, with the new modalities of therapy acting on the biological activity of the tumours draw the attention of most cancer researches nowadays and represent a major focus for clinical oncology practice. For the detection of HER2/neu protein overexpression and gene amplification, immunohistochemistry (IHC) and in-situ hybridisation (ISH) is the recommended techniques, respectively, with high concordance between the two techniques. The current United Kingdom recommendations for HER2/neu testing are either for a two-tier system using IHC with reflex ISH testing in equivocal positive cases, or a one-tier ISH strategy.

AIM: To compare the results of HER2/neu gene status in patients with breast carcinoma obtained by chromogenic in situ hybridisation with those obtained by immunohistochemistry, and to compare these results with hormonal receptors expression by immunohistochemistry and with age of patients.

METHODS: Immunohistochemistry technique was used for evaluation of status of estrogen receptors (ER) and progesterone receptors (PR) and HER2/*neu* protein expression in 448 Iraqi patients with invasive breast carcinoma with different grades and histological types and then chromogenic in situ hybridization (CISH) technique was applied for all scores of HER2/*neu* to detect the gene status and compare the results in all negative, equivocal and positive cases by immunohistochemistry (IHC). The cases were referred from different centres, and IHC and CISH techniques were done in central public health laboratory in Baghdad over 28 months, from July 2013 to November 2015. A comparison of the results was made to find the relationship between HER2/*neu* and hormone receptors status and other clinical parameters like patients age.

RESULTS: The mean age of the study cases was 49.08 years, ranging from 24 to 83 years. Of the 448 cases of breast carcinoma, 44 (9.8%) cases were of score 0 by IHC, none of them (0%) showed HER2/neu gene amplification by CISH. 71(15.8%) cases were of score 1 by IHC, 15 (21.12%) of them showed HER2/neu gene amplification by CISH, all were of low amplification. There were 306 (68.3%) cases of score 2 by IHC, of which 102 (33.33%) cases showed HER2/*neu* gene amplification by CISH, with 79 (25.81%) of them with low amplification and 23 (7.51%) cases with high amplification, while only one case (0.32%) remained in equivocal category. In score 3, all the 27 (6.0%) cases showed gene amplification with 12 (44.44%) cases with low amplification and 15 (55.55) cases with high amplification with overall percentage of gene amplification in score 3 of 100%. There was a significant inverse relationship between hormone receptors (ER and PR) status and HER2/*neu* gene amplification. No significant relationship was found between the patient's age and HER2/*neu* gene amplification.

CONCLUSION: Although immunohistochemistry is a widely used, less expensive and reliable test, we strongly advice performance of chromogenic in situ hybridization in assessment of HER2/neu gene status in all cases diagnosed with breast carcinoma as significant number of cases that were reported as negative by immunohistochemistry showed positive amplification by chromogenic in situ hybridization and can get benefit from anti-HER2 targeted treatments.

Introduction

ERBB2 (HER2/neu), a human epidermal growth factor receptor, is a member of the tyrosine kinases family that is involved in important signalling pathways controlling cell proliferation and tumour growth and survival [1]. Hyperfunction of this receptor, due to gene amplification and protein overexpression, has been reported and evaluated in different types of cancers [2]. In approximately 15% of early invasive breast carcinoma, ERBB2 (HER2/neu) overexpression is present and serve as a poor prognostic marker and an important predictive of response to specific therapy [3]. Tumours with

increased levels of the ERBB2 (HER2/neu) are characterised by more aggressive histological features, more rapid growth rate and a shortened overall survival and relapse-free time, compared with those with no ERBB2 (HER2/neu) overexpression [4].

The great response achieved with anti-ERBB2 (HER2/neu) targeted therapies in ERBB2 (HER2/neu)-positive breast carcinoma cases in addition to serious side effects of these expensive drugs increase the necessity for accurate assessment of ERBB2 (HER2/neu) status [3].

Also, other studies stated that cases with overexpression of ERBB2 (HER2/neu) are associated with impaired responsiveness to tamoxifen (anti-estrogen targeted treatment) in breast carcinoma that is positive for both ERBB2 (HER2/neu) and hormone-receptor [5].

The rapid development of new, clinically advanced information in this subject can induce challenges for oncologists to understand and properly react to these updates [3].

ERBB2 (HER2/neu) status should be determined in all newly diagnosed, recurrent and metastatic breast carcinoma. Bilateral tumours, histologically different ipsilateral tumours or widely separated tumours should each be assessed. Retesting the non-responding stable or progressive ERBB2 (HER2/neu)-negative tumours particularly the high-grade or those with a long interval time between preoperative biopsy and excisional surgery may be considered but are not recommended routinely because of the lack of evidence [3].

By immunohistochemistry, more than 10% complete strong membrane staining defines a positive status of protein overexpression. In situ hybridisation, either bright field chromogenic or fluorescent is used either initially or in immunohistochemistry equivocal positive cases to detect the presence of ERBB2 (HER2/*neu*) gene amplification [3].

Excellent concordance in results between needle core biopsy and surgical specimens has been obtained using immunohistochemistry (IHC) and *in situ* hybridisation (ISH) [6]. In most centers in United Kingdom, ERBB2 (HER2/neu) testing is performed on diagnostic core biopsy specimens, mainly for the results to be available at the time of postoperative multidisciplinary team (MDT) for treatment plan discussion and also to study the use of neoadjuvant treatment which is increasingly given for operable cases with no repeat on excision specimens needed if the test is strongly positive or negative. However; re-performing the assay on incisional or excisional surgical specimens can be considered in the following conditions:

- When the initial ERBB2 (HER2/neu) test result in a core needle biopsy specimen of a primary breast carcinoma is negative, another ERBB2 (HER2/neu) test could be ordered on the excisional specimen if the tumor grade is 3, if small amount of invasive carcinoma is present in the core biopsy specimen, if resection specimen contains carcinoma of higher grade than that in the core, if results of core biopsy is equivocal for ERBB2 (HER2/neu) by both ISH and IHC, or if there is any doubt about handling of the core biopsy specimen (short fixation time, long ischemic time, different fixatives).

- When the initial ERBB2 (HER2/neu) test result in a core needle biopsy specimen of a primary breast carcinoma is positive, another ERBB2 (HER2/neu) test could be ordered on the excisional specimen in histologic grade 1 carcinoma of the following types: ER and PR positive infiltrative ductal or lobular carcinoma, tubular, mucinous, cribriform or adenoid cystic carcinoma. While a new ERBB2 (HER2/neu) test must not be done in previous histologic types if the initial ERBB2 (HER2/neu) test was negative [6], [7].

Cytology specimens from fine needle aspiration of primary breast carcinoma are not ideal for assessment of ERBB2 (HER2/neu) status by IHC as that differentiation between invasive and *in situ* carcinoma cannot be made on these samples. However, if the cytology specimens are the only available material, or in case of metastasis, some studies indicate that ISH is reliable for assessment of ERBB2 (HER2/neu) status in liquid-based and cell block preparations [8].

In the case of bone metastasis, when ERBB2 (HER2/neu) assessment is required, decalcification techniques can detrimentally affect immunohistochemical assessment, and these decalcified specimens should be better tested with ISH techniques [9], [10].

Algorithms to be followed for testing ERBB2 (HER2/neu)

For determination of ERBB2 (HER2/neu) status, IHC for evaluation of protein overexpression and ISH for assessment of gene amplification status are the recommended techniques. High agreement between protein overexpression and gene amplification is reported [4], [11], [12]. The current recommendations in the United Kingdom for ERBB2 (HER2/neu) testing are for “a two-tier system using IHC with reflex ISH testing if required, using the model shown in figure 1, or a one-tier ISH strategy”. In the usual practice for ERBB2 (HER2/neu) testing IHC is used with the analysis of equivocal positive cases by ISH; however, some laboratories use upfront ERBB2 (HER2/neu) ISH testing, especially if the quality of tissue fixation and processing is questionable [13].

ISH has usually been performed using a fluorescence ISH (FISH) technique. Bright-field ISH can be used to assess ERBB2 (HER2/neu) status with a light microscope and is now considered as an acceptable alternative to FISH [14].

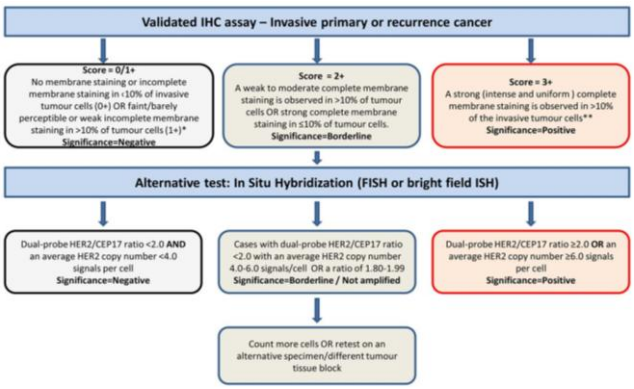

*Figure 1: Recommended ERBB2 (HER2/neu) scoring algorithm for immunohistochemistry (IHC) and in situ hybridisation (ISH); *no sufficient data is available about moderate complete membrane staining in ≤ 10% of tumour cells or strong incomplete membrane staining in > 10% of tumour cells. Repeat on another specimen (tissue block) is advisable; **Membrane staining should be intense and uniform and like chicken-wire. Ignoring incomplete and pale membrane staining in percentage estimation*

The most widely used bright field ISH uses a DNA probe coupled to a silver ISH or chromogenic ISH detection system, or a combination of both [14]. ISH can be performed using either a single probe to enumerate ERBB2 (HER2/neu) copies per nucleus or a dual-probe technique that allows determination of the ERBB2 (HER2/neu): CEP17 ratio and ERBB2 (HER2/neu) gene copy number. Because of this, the use of a chromosome 17 probe is strongly advocated [3].

IHC is a semi-quantitative method for analysis of HER2/neu protein expression, and it is quick, easy and less expensive; however, it is more subjective and susceptible to discrepancies in test results with different laboratory parameters [15]. ISH is a quantitative method for analysis of HER2/neu gene copy number; however, it is more time consuming and costly but more reliable because of its quantitative nature [16], [17]. The choice of most suitable test for HER2/neu status is still an area of discussion with various studies showing and evaluating different opinions regarding the gold standard test algorithm to be followed [18].

Chromogenic in situ hybridisation (CISH)

CISH assays depend on a technique named subtractive hybridisation that uses a DNA probe visualised by a peroxidase reaction. CISH is a new technique that uses a chromogen-labelled probe which offers several advantages. First, simultaneous assessment of tissue morphology and CISH signals at high power using a light microscope. Second, by using the dual probe method, two targets can be detected at the same time. Third, high contrasting between distinct red and green signals. Fourth, it allows quick and easy interpretation of results as compared to IHC, and it is standardised with complete kits and no need for costly fluorescent microscope [19].

Fixation and processing of the specimens for HER2/neu assessment

Proper fixation of specimens used for HER2/neu testing must be ensured and the cold ischemic time (the time between removal of the tissue from the patient and its placing in fixative) should be as short as possible, typically less than one hour [20]. Formalin-fixed, paraffin-embedded tumour tissue samples are suitable for the assay. Tumour samples must be fixed in buffered formalin and embedded in paraffin wax; fixatives that contain alcohol will result in staining of normal tissue and the use of Bouin's fixative will prevent testing by fluorescence in situ-based methods. Other methods of fixation can also badly affect antigen reactivity [3].

At least six hours of fixation are recommended for the core biopsies. Surgical specimens have to be incised as near as possible through the carcinoma to allow good penetration of the fixative and then dissected into 5 – 10 mm slices to ensure rapid penetration and fixation. Tissue must be placed in an adequate volume (typically 1:10; tissue: fixative) of fixative for at least twenty-four hours and not more than three days. Centres that use rapid fixation and processing methods should validate their methodology for HER2/neu assessment [3].

Sections must be stained within one to two days of cutting and drying. Excessive section drying time will lead to loss of HER2/neu expression, and therefore, it is recommended that freshly cut sections are either dried at 60°C for one hour or 37°C overnight [21].

Material and Methods

Immunohistochemistry technique was used for evaluation of ER, PR status and HER2/neu protein expression in 448 Iraqi patients with invasive breast carcinoma with different grades and histological types and then CISH technique was applied for all scores of HER2/neu to detect the gene status and compare the results with all 115 (25.6%) negative, 306 (68.3%) equivocal and 27 (6.0%) positive cases by immunohistochemistry. The cases were referred from different centres, and IHC and CISH techniques were done in central public health laboratory in Baghdad over three years, from July 2013 to November 2015. A comparison of the results was made to find the relationship between HER2/neu and hormone receptors status and other clinical parameters like patients age. IHC test kit was provided by (Dako, Glostrup, Denmark) and the CISH test kit was provided by Zytovision medical company, ZytoDot ® 2C SPEC ERBB2/CEN 17 Probe.

IHC for ER, PR and HER 2

Multiple sections of (3-4 µm) thickness were cut from breast carcinoma tissue blocks and placed on positively charged slides to overnight incubation at 56ºC. Sections were de-paraffinized via xylene and further rehydrated with graded alcohols to distilled water. After blocking endogenous peroxidase activity with 3% hydrogen peroxide in methanol, antigen retrieval was achieved through heating the slides in 10 mmol/l citrate buffer (pH 6) by using a water bath. Rabbit monoclonal anti-HER-2/*neu* primary antibody (Dako, Glostrup, Denmark) were applied for 60 minutes at dilution of 1:800. The Envision Kit (Dako) was used for the application of the secondary antibody. Signals were developed with Diaminobenzidine (DAB) followed by light nuclear counterstaining by haematoxylin. Each test was run with a known positive and negative control.

For evaluating the immunohistochemistry for the ERBB2 antibody, ASCO/CAP guidelines [6]. Were followed, as shown in Table 1.

Table 1: Interpretation of results of immunohistochemistry for the ERBB2 antibody

Staining pattern	Score	HER2/neu protein overexpression assessment
No membrane staining or incomplete membrane staining in < 10% of the invasive tumour cells	0	Negative
A weak to moderate complete membrane staining is observed in > 10% of tumour cells OR strong complete membrane staining in ≤10% of tumour cells	1+	Negative
A weak to moderate complete membrane staining is observed in > 10% of tumour cells OR strong complete membrane staining in ≤10% of tumour cells	2+	Weakly positive/equivocal
A strong (intense and uniform) and complete membrane staining in > 10% of invasive tumour cells	3+	Strong positive

For estrogen and progesterone receptor detection, Heat Induced Epitope Retrieval (HIER) was achieved through heating the slides in EDTA buffer (pH 9) for 25 minutes at 95ºC. Primary antibody clones used for ER and PR were DAKO 1D5 (1:200) and DAKO PR 88 (1:200) respectively.

Table 2: Allred system of scoring for estrogen and progesterone receptors

Score	Intensity of staining
0	Negative
1	Weak
2	Intermediate
3	Strong
Score	Percentage of stained cells
0	No cells are ER/PR positive
1	< 1% cells are ER/PR positive
2	1-10% of cells are ER/PR positive
3	11-33% of cells are ER/PR positive
4	34-66% of cells are ER/PR positive
5	67-100% of cells are ER/PR positive
Allred score (intensity + percentage)	Effect of hormone therapy
0-1	No effect
2-3	Small (20%) chance of benefit
4-6	Moderate (50%) chance of benefit
7-8	Good (75%) chance of benefit

Interpretation of results was made as per ER/PR reporting guidelines of the Allred scoring system depending on the proportion of stained cell (PS), given score of 0-5 , and intensity of staining (IS), given score of 0-3, in a combined score (AS), from 0/8 to 8/8, results from their summation as shown in table 2 [21]. Proper controls were used as indicated.

CISH HER2/neu

The ZytoDot ® 2C SPEC ERBB2/CEN 17 probe was designed for the simultaneous detection of ERBB2 and centromere 17 in formalin-fixed, paraffin-embedded tissue sections or cell samples.

Probe Description

The Zyto*Dot*® 2C SPEC ERBB2/CEN 17 probe is a mixture of a Digoxigenin-labeled probe specific for the ERBB2 gene at 17q12 and a Dinitrophenyl-labeled CEN 17 probe specific for the alpha satellite centromeric region of chromosome 17 (D17Z1). Using the ZytoDot® 2C SPEC ERBB2/CEN 17 Probe Kit, two green (ERBB2) and two red (CEN 17) signals are expected in a normal interphase nucleus. In a cell with amplification of the ERBB2 gene locus, multiple copies of the green signal or green signal clusters will be observed. The results interpretation followed the manufacturer instructions of ZytoDot 2C SPEC HER2/CEN 17 Probe Kit.

The UK recommendation when using dual probe CISH is to report the HER2/CEP17 signal ratio and HER2/neu copy number [3].

Tumours with a HER2/CEP17 signal ratio ≥ 2.0 and/or a mean HER2/neu gene, copy number ≥ 6 are said to be positive. Cases with a HER2/CEP17 signal ratio < 2.0 with an average HER2/neu copy number < 4.0 signals/cells are said to be negative. The controversy is still present about cases with HER-2/*neu* gene copy number ≥ 6, but the HER2/CEP17 ratio is < 2 [6]. Selection and assessment of normal breast tissue cells to confirm proper hybridisation, successful detection and visualisation, before testing of the invasive carcinoma were done. The numbers of HER2/neu and chromosome 17 signals were scored and recorded, and the mean HER2/neu to chromosome 17 copy ratio is assessed in 20 – 60 cells, where possible, using at least three distinct tumour fields. In cases where either obvious amplification is observed, or the ratio is below 1.5, scoring of just 20 tumour cells is sufficient.

Cells to be assessed and scored are only those with identifiable nuclear borders. Over-damaged, digested, or truncated nuclei were excluded. Only cells with minimum one copy of HER2/neu and CEP17 were scored. In cases with tumour heterogeneity (coexistence of different population of tumour cells having distinct HER2/neu amplification patterns within the same tumour), or the ratio is close to 2.0, or the average copy number is between ≥ 4.0 and < 6.0 signals/cell, additional cells were scored (at least 40).

Table 3: Evaluation of HER2/neu gene status using chromogenic in situ hybridisation

Amplification	Her-2/*neu* gene status
High-level	> 10 copies or large cluster of amplicon per nucleus in > 50% of cancer cells
Low-level	6–10 copies or small cluster of amplicon per nucleus in > 50% of cancer cells
None	1–5 copies per nucleus of cancer cells

Results

Patients' age

The mean age of the 448 cases of invasive breast carcinoma studied in this study was 49.08 years, ranging from 24 to 83 years old.

Estrogen and Progesterone receptors status by IHC

Expression of estrogen receptors was seen in 279 (62.27%) cases. 66 (14.7%) were with weak expression (AS score 2-3), 143 (31.9%) were with moderate expression (AS score 4-6) and 70 (15.6%) with strong expression (AS score 7-8). Expression of progesterone receptors was seen in 304 (67.85%) cases. Forty-nine (10.9%) were with weak expression (AS score 2-3), 151 (33.7) were with moderate expression (AS score 4-6) and 104 (23.2%) with strong expression (AS score 7-8). As in Table 4.

Table 4: Estrogen and Progesterone receptors status by IHC

Hormone receptors expression	NO expression (AS score 0-1)	Weak expression (AS score 2-3)	Moderate expression (AS score 4-6)	Strong expression (AS score 7-8)
ER status	169 (37.7%)	66 (14.7%)	143 (31.9%)	70 (15.6%)
PR status	144 (32.1%)	49 (10.9%)	151 (33.7)	104 (23.2%)

HER2/neu protein overexpression by IHC

HER2/neu protein overexpression was positive (score + 3) in 27 (6.0%) cases, equivocally positive (score + 2) in 306 (68.3%) cases and negative (score + 1/0) in 115 (25.6%) cases of the studied specimens. As in Table 5.

Table 5: HER2/neu protein overexpression by IHC

HER2/neu score	No. of the cases	A percentage of the total number
Score 0	44	9.8%
Score + 1	71	15.8%
Score + 2	306	68.3%
Score + 3	27	6.0%

CISH results for HER2/neu gene amplification

HER2/neu gene amplification was seen in 144 (32.14%) specimens of the 448 studied cases. HER2/neu gene was not amplified in all cases (0%) of score 0 by IHC, while all the 27 cases (100%) with score +3 by IHC showed gene amplification by CISH, 12(44.4%) cases showed low amplification and 15 (55.5%) cases showed high amplification. Within cases of score + 1 by IHC, 15 (21.1%) cases show low gene amplification and no case show high amplification.

Table 6: CISH results for HER2/neu gene amplification

Ihc Results for HER2/neu Protein Over-Expression	Cish Results for HER2/neu Gene Amplification								
	Total No. of cases	Not amplified	Percentage (%)	Low amplification	Percentage (%)	High amplification	Percentage (%)	Equivocal	Percentage (%)
Score 0	44	44	100%	0	0%	0	0%	0	0%
Score +1	71	56	78.8%	15	21.1%	0	0%	0	0%
Score +2	306	203	66.3%	79	25.8%	23	7.5%	1	0.3%
Score +3	27	0	0%	12	44.4%	15	55.5%	0	0%

Among equivocal positive cases of score + 2 by IHC, 102 (33.3%) cases show HER2 gene amplification, 79 (25.8%) cases with low amplification and 23 (7.5%) cases with high amplification while one (0.3%) case was equivocal. As in Table 6 and Table 7.

Table 7: Percentage of positive cases for HER2/neu gene amplification by CISH within each score

HER2/neu protein expression by IHC	Percentage of positive cases for HER2/neu gene amplification by CISH within each score
Negative score 0	0 %
Negative score 1	21.1 %
Equivocal positive score 2	33.3 %
Positive score 3	100 %

Relationship between HER2/neu and Estrogen receptor

HER2/neu gene amplification was present in 70 (41.4%) cases of those with no estrogen receptor expression, while 75 (26.9%) cases of those expressing estrogen receptors showed no HER2/neu gene amplification. There is a significant negative relationship between HER2/neu status and ER expression with a p-value of 0.015. As shown in Table 8.

Table 8: Relationship between HER2/neu and Estrogen receptors

Relationship between HER2/neu and Estrogen receptor		CISH results				P value
		Not amplified	percentage	Amplified	percentage	
ER status	No expression	99	58.6%	70	41.4%	0.015
	expression	204	73.1%	75	26.9%	

Relationship between HER2 and Progesterone receptor

HER2/neu gene amplification was present in 64 (44.4%) cases of those with no progesterone receptor expression, while 81 (26.6%) cases of those expressing progesterone receptors, showed no HER2/neu gene amplification.

There is a significant negative relationship between HER2/neu status and PR expression with a p-value of 0.001. As shown in Table 9.

Table 9: Relationship between HER2 and Progesterone receptor

Relationship between HER2/neu and Progesterone receptor		CISH results				P value
		Not amplified	percentage	Amplified	percentage	
PR status	No expression	80	55.5%	64	44.4%	0.001
	expressed	223	73.3%	81	26.6%	

Relationship between HER2/neu and patients age

HER2/neu gene was amplified in 67 (36.4%) specimens from young patients (45 years and younger), and it was amplified in 78 (29.5%) specimens from old patients (more than 45 years) with no significant relationship between patient's age and HER2/neu gene amplification. As shown in Table 10.

Table 10: Relationship between HER2 and patients age

Relationship between HER2/neu and patients age		CISH results				P value
		Not amplified	percentage	Amplified	percentage	
Patients age	≤ 45 years	117	63.6%	67	36.4%	0.126
	> 45 years	186	70.5%	78	29.5%	

Discussion

In Iraq, breast cancer is the most frequent cancer in females with 4824 cases were reported during 2015, with an annual incidence of 13.3 per 100000 population [23].

Overexpression of human epidermal growth factor receptor type 2 (HER2/neu or ErbB-2), a 185-kD receptor first described three decades ago, occurs in many tumours like 48.38% of endometrioid endometrial carcinoma and 20% to 30% of invasive breast carcinomas [24], [25]. Generally, patients with carcinomas that overexpress HER2/neu or that have a high copy number of its gene have worsened overall survival and might have different responses to a variety of hormonal and chemotherapeutic agents [26], [27], [28]. Thus, therapies aimed to target HER2/neu appear to be crucial in treating breast cancer. One such treatment is trastuzumab (Herceptin, Genentech), which is a humanised monoclonal antibody. Trastuzumab binds to an extracellular juxtamembrane domain of HER2/neu and inhibits proliferation and survival of HER2/neu-dependent tumours. It has been approved by the Food and Drug Administration (FDA) for patients with invasive breast carcinoma with HER2/neu overexpression. The choice of a standard gold method for testing HER2/neu status had been an area of argument for a long time with controversy regarding the use of immunohistochemistry with the application of in situ hybridisation in equivocal cases or the use of in situ hybridisation from the start in all cases diagnosed with invasive breast carcinoma [13].

In this study, IHC was done to ascertain the score of HER2/neu protein expression in tumour samples. About two thirds of the cases (306; 68.3%) were equivocally positive (2+ reactivity) whereas 115 (25.6%) case were negative (0, 1+ reactivity) and 27 (6.0%) cases were positive (3+ reactivity).

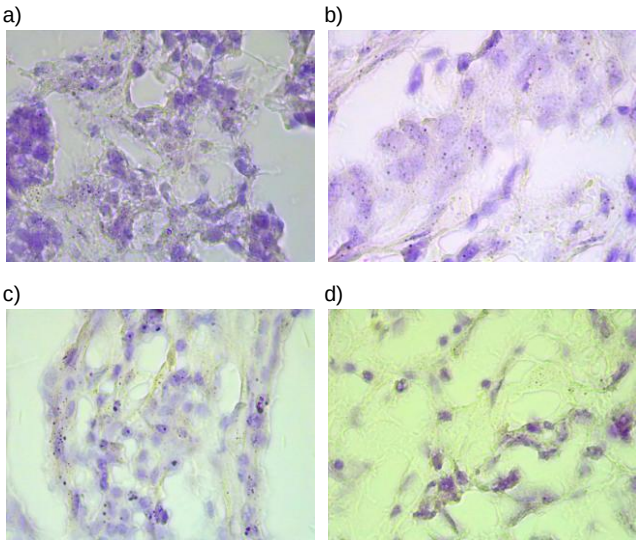

Figure 2: a) HER2/neu high amplification by CISH in the equivocal case by IHC; b) HER2/neu low amplification by CISH in the equivocal case by IHC; c) HER2/neu equivocal by CISH in the equivocal case by IHC; d) HER2/neu non-amplification by CISH in the equivocal case by IHC

These figures differ from that reported in other studies like Eswarachary V et al., that showed approximately a third of the cases (143/432, 33.10%) being positive (3+ reactivity) while 47 cases (10.88%) were negative (0, 1+ reactivity) with a substantial number of cases (242/432; 56.01%) showed equivocal positivity (2+ reactivity) [29]. In Varga Z et al., study, 12% of the cases were 3+ on IHC (184 of 1522 cases), 26.5% of the cases were 2+ (404 of 1522), and 61.4% of the cases were negative 0/1+ on IHC (934 of 1522) [30]. This may be attributed to sampling bias as most of the cases in this study were of score 2+ that are routinely submitted for in situ hybridisation to test the HER2/neu gene amplification.

Expression of estrogen receptors was seen in 279 (62.27%) cases in this study, and the expression of progesterone receptors was seen in 304 (67.85%) cases. This is in agreement with Eswarachary V et al., where approximately two thirds (284/432, 65.74%) of their cases were positive for ER receptor expression whereas 59.72% (258/432) of the cases were positive for PR receptor expression [29]. The same results were obtained by Alwan N et al., showing the registered rates of positive ER and PR tumour contents of 67.8% and 65.3% respectively [31].

Analysing the correlation between CISH HER2/neu status and ER/PR expression demonstrated that ER and PR expressions were

higher in HER2/neu negative tumours compared to HER2/neu positive tumours. These inverse associations between the expression of ER/PR and HER2/neu amplification were significant with p-value 0.015(with ER) and p-value 0.001(with PR). This is similar to what was obtained by Eswarachary V et al., but with a still significant number of HER2/neu positive tumours, which also expressed ER/ PR [29]. The inverse relationship between ER/PR status and HER2/neu is logic in that HER2/neu hyper-functioning usually associates with high-grade aggressive tumours in contrary to ER and may explain the cause of poor responsiveness to tamoxifen therapy in a patient who express both ER and HER2/neu [29].

Regarding the correlation between age of the patient and HER2/neu status by CISH, there was no significant correlation between them with p-value 0.126. This is in agreement with Barros et al., [32], while Jabbar N et al., found in their study significant correlation between female age and her HER2/neu amplification by CISH; where increased age was associated more with gene amplification [33].

HER2/neu gene amplification rates in different scores of HER2/neu protein expression by IHC in this study were as follow: 0% in score 0, 21.1% in score 1+ (all were with low amplification), 33.3% in score 2+ (25.8% with low amplification and 7.5% with high amplification with one (0.3%) case equivocal) and 100% in score3+. In a study done by Vocaturo et al., there was no case of 0/1+ by IHC score showed gene amplification by CISH, 53% of score 2+ showed gene amplification and 64% of cases with score 3+ were amplified by CISH [34]. In another study from Iraq done by Khashman B et al., for equivocally positive cases only, 24.5% (12 out of 49) showed low amplification results while 8.1% (4 out of 49) showed high amplification results with 32.6% overall percentage of CISH positive cases within score 2+ [35]. Manuelito A Madrid et al., in their study of 160 cases of invasive breast carcinoma for HER2/neu status, 80 were IHC positive (score 2+ and 3+), and 80 were IHC negative (score 0 and score 1+). With the CISH assay, 58 (36.25%) of their 160 cases showed HER2/neu gene amplification. No case (0%) of the negative ones show gene amplification by CISH, while all cases (100%) of score 3+ showed positive results with CISH, 9 (22.5%) cases with low amplification and 39 (97.5%) cases with high amplification, 18 cases (45%) of score 2+ were positive by CISH, 8 (20%) cases with low amplification and 10 (25%) cases with high amplification [36]. The subjectivity of the immunohistochemical method for assessment of HER2/neu expression lies behind this discrepancy in results between different studies. This may result from up scoring or down the scoring of the expression, so that cases with score 1+ may be reported by others as 2+ or underestimate score 3+ as 2+. For that reason, the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP), human epidermal growth factor receptor 2 (HER2) testing Expert Panel aimed to improve the analytic validity of HER2/neu testing and the clinical utility of HER2/neu as a predictive biomarker for the potential responsiveness to therapies targeting the HER2/neu protein [37]. Eswarachary V et al., in their similar research, but with use of fluorescent in situ hybridisation (FISH) technique for HER2/neu gene status testing, HER2/neu was non-amplified in more than half of their cases (223/432, 51.6%) while it was amplified in 46.3% cases (200/432). IHC was done to evaluate the expression of HER2/neu protein in tumour samples. Approximately one-third of the samples (143/432, 33.1%) were positive (3+ reactivity), whereas 47 samples (10.8%) were negative (0/1+ reactivity). Also, a substantial number of patients (242/432, 56%) showed equivocal 2+ reactivity. Of the equivocal IHC cases, 68 (28.10%) cases were amplified for HER2/neu by FISH, 168 (69.42%) cases were not amplified, and the rest (2.5%) were FISH equivocal. Within score 3+, 91.6% of the cases showed amplification by FISH and, within score 0/1+, only 2.1% of the cases were positive [29].

Study Limitations: The pre-analytical variables, including fixation time, environment and tissue processing, could be controlled because the significant number of samples was referred from other centres.

In conclusion, although IHC is a widely used, less expensive and reliable test, we strongly advice performance of CISH in assessment of HER2/nu gene status in all cases diagnosed with breast carcinoma as significant number of cases that were reported as negative by IHC showed positive amplification by CISH and can get benefit from anti-HER2 targeted treatments. In addition to the subjectivity and the several limitations in the interpretation of the IHC test results.

References

1. Arteaga CL, Sliwkowski MX, Osborne CK, Perez EA, Puglisi F, Gianni L. Treatment of HER2-positive breast cancer: current status and future perspectives. Nature Reviews Clinical Oncology. 2011; 9: 16-32. https://doi.org/10.1038/nrclinonc.2011.177 PMid:22124364

2. Gravalos C, Jimeno A. HER2 in gastric cancer: a new prognostic factor and a novel therapeutic target. Annals of Oncology. 2008; 19:1523-29. https://doi.org/10.1093/annonc/mdn169 PMid:18441328

3. Rakha EA, Pinder SE, Bartlett JM, Ibrahim M, Starczynski J, Carder PJ, Provenzano E, Hanby A, Hales S, Lee AH, Ellis IO. Updated UK Recommendations for HER2 assessment in breast cancer. Journal of clinical pathology. 2015; 68(2):93-9. https://doi.org/10.1136/jclinpath-2014-202571 PMid:25488926 PMCid:PMC4316916

4. Pauletti G, Dandekar S, Rong H. Assessment of methods for tissue-based detection of the HER-2/neu alteration in human breast cancer: a direct comparison of fluorescence in situ

hybridization and immunohistochemistry. J Clin Oncol. 2000; 18:3651-64. https://doi.org/10.1200/JCO.2000.18.21.3651 PMid:11054438

5. Elledge RM, Green S, Ciocca D. HER-2 expression and response to tamoxifen in estrogen receptor-positive breast cancer: a Southwest Oncology Group Study. Clin Cancer Res. 1998; 4:7-12.

6. Wolff AC, Hammond ME, Hicks DG. 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. 2013; 31:3997-4013. https://doi.org/10.1200/JCO.2013.50.9984 PMid:24101045

7. Greer LT, Rosman M, Mylander WC. Does breast tumor heterogeneity necessitate further immunohistochemical staining on surgical specimens? J Am Coll Surg. 2013; 216:239-51. https://doi.org/10.1016/j.jamcollsurg.2012.09.007 PMid:23141136

8. Durgapal P, Mathur SR, Kalamuddin M. Assessment of her-2/neu status using immunocytochemistry and fluorescence in situ hybridization on fine-needle aspiration cytology smears: Experience from a tertiary care centre in india. Diagn Cytopathol. 2014; 42:726-31. https://doi.org/10.1002/dc.23088 PMid:24376261

9. Zustin J, Boddin K, Tsourlakis MC. HER-2/neu analysis in breast cancer bone metastases. J Clin Pathol. 2009; 62:542-6. https://doi.org/10.1136/jcp.2008.059717 PMid:19474354

10. Penault-Llorca F, Coudry RA, Hanna WM. Experts' opinion: recommendations for retesting breast cancer metastases for HER2 and hormone receptor status. Breast. 2013; 22:200-2. https://doi.org/10.1016/j.breast.2012.12.004 PMid:23352656

11. Walker RA, Bartlett JM, Dowsett M. HER2 testing in the UK: further update to recommendations. J Clin Pathol. 2008; 61:818-24. https://doi.org/10.1136/jcp.2007.054866 PMid:18381380

12. Press MF, Sauter G, Bernstein L. Diagnostic evaluation of HER-2 as a molecular target: an assessment of accuracy and reproducibility of laboratory testing in large, prospective, randomized clinical trials. Clin Cancer Res. 2005; 11:6598-607. https://doi.org/10.1158/1078-0432.CCR-05-0636 PMid:16166438

13. Sauter G, Lee J, Bartlett JM. Guidelines for human epidermal growth factor receptor 2 testing: biologic and methodologic considerations. J Clin Oncol. 2009; 27:1323-33. https://doi.org/10.1200/JCO.2007.14.8197 PMid:19204209

14. Arnould L, Roger P, Macgrogan Gl. Accuracy of HER2 status determination on breast core-needle biopsies (immunohistochemistry, FISH, CISH and SISH vs FISH). Mod Pathol. 2012; 25:675-82. https://doi.org/10.1038/modpathol.2011.201 PMid:22222637

15. Olsson H, Jansson A, Holmlund B, Gunnarsson C. Methods for evaluating HER2 status in breast cancer: comparison of IHC, FISH, and real-time PCR analysis of formalin-fixed paraffin-embedded tissue. Pathology and Laboratory Medicine International. 2013; 5:31-37. https://doi.org/10.2147/PLMI.S44976

16. Rosa FE, Santos RM, Rogatto SR, Domingues M. Chromogenic in situ hybridization compared with other approaches to evaluate HER2/neu status in breast carcinomas. Brazilian Journal of Medical and Biological Research. 2013; 46:207-16. https://doi.org/10.1590/1414-431X20132483 PMid:23558859 PMCid:PMC3854374

17. Kaufman PA, Bloom KJ, Burris H, Gralow JR, Mayer M, Pegram M, et al. Assessing the discordance rate between local and central HER2 testing in women with locally determined HER2-negative breast cancer. Cancer. 2014; 120:2657-64. https://doi.org/10.1002/cncr.28710 PMid:24930388 PMCid:PMC4232097

18. Allred DC. Issues and updates: evaluating oestrogen receptor, progesterone receptor, and HER2 in breast cancer. Modern Pathology. 2010; 23:S59. https://doi.org/10.1038/modpathol.2010.55 PMid:20436503

19. Reliable and simple detection of genomic alterations using light microscopy. ZytoDotR 2CTM - 2- Color CISH for the detection of genomic alterations. A user manual provided by ZytoVision GmbH - Fischkai 1, 27572 Bremerhaven- Germany. ZytoVision molecular diagnostics simplified, 2019:182-3. www. Zytovision.com

20. Lee AH, Key HP, Bell JA. The effect of delay in fixation on HER2 expression in invasive carcinoma of the breast assessed with immunohistochemistry and in situ hybridisation. J Clin Pathol. 2014; 67:573-5. https://doi.org/10.1136/jclinpath-2013-201978 PMid:24737400

21. Lundgaard Hansen B, Winther H, Moller K. Excessive section drying of breast cancer tissue prior to deparaffinisation and antigen retrieval causes a loss in HER2 immunoreactivity. Immunocytochemistry. 2008; 6:117-22.

22. Allred DC, Bustamante MA, Daniel CO, Gaskill HV, Cruz AB Jr. Immunocytochemical analysis of estrogen receptors in human breast carcinomas. Evaluation of 130 cases and review of the literature regarding concordance with biochemical assay and clinical relevance. Arch Surg. 1990; 125:107-13. https://doi.org/10.1001/archsurg.1990.01410130113018 PMid:1688490

23. Iraqi cancer registry 2015 (page 29). Ministry of Health and Environment/Iraq- Iraqi Cancer Board, 2015. Accessed at: https://moh.gov.iq/upload/upfile/ar/833.pdf

24. Abd Ali Z, Hassan F, Yahya A. Immunohistochemical expression of HER2/neu receptors in Iraqi patients with endometrioid carcinoma. Journal of Clinical and Diagnostic Research. 2018, 12(11):5-8.

25. Schechter AL, Stern DF, Vaidyanathan L. The neu oncogene: an erb-B-related gene encoding a 185,000-Mr tumour antigen. Nature. 1984; 312:513-516. https://doi.org/10.1038/312513a0 PMid:6095109

26. Albanell J, Bellmunt J, Molina R. Node-negative breast cancers with p53 (-)/HER2-neu (-) status may identify women with very good prognosis. Anticancer Res. 1996; 16:1027-32.

27. Ellis MJ, Coop A, Singh B. Letrozole is more effective neoadjuvant endocrine therapy than tamoxifen for ErbB-1- and/or ErbB-2-positive, estrogen receptor-positive primary breast cancer: evidence from a phase III randomized trial. J Clin Oncol. 2001; 19:3808-16. https://doi.org/10.1200/JCO.2001.19.18.3808 PMid:11559718

28. Berry DA, Muss HB, Thor AD. HER-2/neu and p53 expression versus tamoxifen resistance in estrogen receptor-positive, node-positive breast cancer. J Clin Oncol. 2000; 18:3471-79. https://doi.org/10.1200/JCO.2000.18.20.3471 PMid:11032587

29. Eswarachary V, Mohammed I, Jayanna P, PatilOkaly G, Nargund A, Dhondalay G. HER2/neu testing in 432 consecutive breast cancer cases using FISH and IHC - A comparative study. Journal of Clinical and Diagnostic Research. 2017, 11(4):1-5. https://doi.org/10.7860/JCDR/2017/25625.9521 PMid:28571140 PMCid:PMC5449786

30. Varga Z, Noske A, Ramach C, Padberg B and Moch H. Assessment of HER2 status in breast cancer: overall positivity rate and accuracy by fluorescence in situ hybridization and immunohistochemistry in a single institution over 12 years: a quality control study. BMC Cancer. 2013; 13:615. https://doi.org/10.1186/1471-2407-13-615 PMid:24377754 PMCid:PMC3879657

31. Alwan N, Tawfeeq F, Muallah F. Breast cancer subtypes among Iraqi patients: identified by their Er, Pr and Her2 Status. J Fac Med Baghdad. 2017; 59(4):303-07. https://doi.org/10.32007/med.1936/jfacmedbagdad.v59i4.6

32. Barros FF. Biological characterisation of HER2 amplified breast cancer (Doctoral dissertation, University of Nottingham).

33. Jabbar N, Altaee M, Naji R. Correlation of Her-2/neu gene amplification by FISH and CISH with clinicopathological parameters of Iraqi breast carcinoma patients at central public health laboratories. Bio--Genetics Journal. 2016; 4(4):73-77.

34. Vocaturo A, Novelli F, Benevolo M, Piperno G, Marandino F, Cianciulli A, et al. Chromogenic in situ hybridization to detect HER-2/neu gene amplification in histological and thin prep®-processed

breast cancer fine-needle aspirates: A sensitive and practical method in the trastuzumab era. The Oncologist. 2006; 11:878-86. https://doi.org/10.1634/theoncologist.11-8-878 PMid:16951391

35. Khashman B, Abdul-Sattar S. Determination of HER2 gene amplification using chromogenic in situ hybridization (CISH) in Iraqi patients with breast carcinoma. International Journal of Science and Research (IJSR). 2017; 6(8).

36. Manuelito A Madrid and Raymundo W Lo. Chromogenic in situ hybridization (CISH): a novel alternative in screening archival breast cancer tissue samples for HER-2/neu status. Breast Cancer Research. 2004; 6(5):1. https://doi.org/10.1186/bcr915 PMid:15318940 PMCid:PMC549176

37. Wolff AC, Hammond ME, Hicks DG. 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. Arch Pathol Lab Med. 2014; 138:241-56. https://doi.org/10.5858/arpa.2013-0953-SA PMid:24099077 PMCid:PMC4086638

38. Goud KI, Dayakar S, Vijayalaxmi K, Babu SJ, Reddy PV. Evaluation of HER-2/neu status in breast cancer specimens using immunohistochemistry (IHC) & fluorescence in-situ hybridization (FISH) assay. Indian J Med Res. 2012; 135(3):312-7.