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Assessment of Her2/Neu status by Silver Insitu Hybridization in Immunohistochemistry Equivocal Cases of Invasive Breast Cancer-Cross Sectional Study in a Sample of Iraqi Patient

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Original Article

Summary

Determination of the Her-2/neu gene amplification status is crucial in breast cancer because it allows selecting patients who would benefit from treatment where the treatment agent designed to recognize and bind to HER2 protein and according to published clinical studies.This study aimed to assess Her2/Neu status by Silver insitu hybridization in immunohistochemistry equivocal cases of invasive breast carcinoma. We conducted this cross-sectional during the period from January to August 2021. The study included 50 Iraqi women with proved diagnosed invasive breast cancer with equivocal IHC result for Her2\Neu. Scoring of Her2 gene amplification was classified according to American society of clinical oncology college of American pathologist(ASCOS CAP) 2018 guidline the obtained results where correlated with given data of patient age and hormone receptor(ER,PR) status. Results showed a mean age of patients of 47.2 ± 9.9 (range: 30 – 75) years. Ten patients (20%) were ER-, PR- and (80%) were ER+, PR+. Among the 50 cases, (62%) HER2/neu was not amplified. On both univariate and binary regression analysis,HER2/neu status was significantly associated with ER-, PR- independent of age , (OR=5.13, P. value = 0.035). In conclusions, Silver insitu hybridization is useful in assessment of HER2/ neu status in immunohistochemistry equivocal cases of invasive breast carcinoma. ER negative ,PR negative status was significantly associated with amplification of HER2/neu independent of patients' age .

Keywords: *Breast cancer, Hormonal Receptors, HER2/neu, Silver insitu hybridization, immunohistochemistry*

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1. INTRODUCTION

Breast cancer is the most common cancer among women worldwide, both in developed and developing countries, accounting for 24% of new cancer cases and 15% of cancer deaths in women (3–5) In Iraq, the annual incidence of breast cancer has increased significantly with an average incidence rate of 37.9/100000 in 2019. The the age-adjusted incidence rate in Iraq found to be greater than that in other countries like Turkey, Iran, Saudi Arabia and Bahrain(6,7). There are many risk factors for breast cancer such as female gender, older age, late age of menopause , contraceptive hormonal methods, benign breast lesions, obesity, smoking and others (8). Screening, early detection and diagnosis of breast cancer play a crucial role in success of treatment of breast cancer (9,10). Earlier studies and literatures documented that Human epidermal growth factor receptor 2(Her 2) overexpressing breast cancer is known to be more aggressive disease and associated with poor prognosis. From other point of view, Her2/neu overexpression considered as predictor of response to endocrine chemotherapy (11,12). On the other hand, neoadjuvant chemotherapy for breast cancer have shown to result in alteration in HER2/neu status by immunohistochemistry, but they have stable status of gene amplification by fluorescence in situ hybridization (FISH). Determination of HER-2/neu oncogene amplification has become necessary for selection of breast cancer patients for trastuzumab (Herceptin) therapy. Fluorescence in situ hybridization (FISH) is currently regarded as a gold standard method for detecting HER-2/neu amplification, but it is not very practical for routine histopathological laboratories. We evaluated a new modification of in situ hybridization, the Silver in situ hybridization (SISH), which enables detection of HER-2/neu gene copies with conventional peroxidase reaction. Clinical importance of HER-2 diagnostics got more attention and awareness by clinician and oncologist with the increased new anti-cancer treatment, HER-2 assays are now considered a substantial an part of diagnostic methods of breast cancer paralleled to hormonal receptors(15). The earliest studies of HER-2 used Southern and Western blotting for detection of HER-2 gene amplification and protein overexpression. These methods are not well suited for routine diagnostics and have been replaced by immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH). A vast majority of HER-2 studies has been done using IHC, which detects the HER-2 protein overexpression on the cell membrane. Without HER-2

oncogene amplification, the protein expression is low and undetectable by IHC. However, IHC is subject to a number of technical artifacts and sensitivity differences between different antibodies and tissue pretreatments. 3 Standardized reagent kits have recently been introduced (such as HercepTest), but mixed results have been reported from their methodological comparisons (16,17). FISH quantifies the number of gene copies in the cancer cell nucleus. Since initial applications to detect HER-2 amplification by FISH, a number of reports have verified its accuracy both in freshly frozen and paraffin-embedded tumor material. FISH is done either using single-color (HER-2 probe only, DAKO, Copenhagen, Denmark) or as a dual-color hybridization (using HER-2 and chromosome 17 centromere probes simultaneously), the latter making it easier to distinguish true HER-2 amplification from chromosomal aneuploidy. FISH from entire cells (cultured cells, pulverized tissue, or imprint touch specimens from tumors) is considered straightforward, but the use of tissue sections complicates the quantitative nature of FISH because of nuclear truncation (slicing) (18,19). The main difficulty for adopting FISH in clinical diagnostics is the need to use fluorescence microscopy, which is not done in most routine diagnostic laboratories. Evaluation of FISH requires a modern epifluorescence microscope equipped with high-quality ×60 and ×100 oil immersion objectives and multi-bandpass fluorescence filters. Moreover, because the fluorescence signals fade within a few weeks, the hybridization results must be recorded with expensive digital cameras(18,19).

Principles of HER2 testing (20–22).

There are a large number of methods available for the detection and measurement of HER2/neu expression; some are based on the overexpression of the protein, with the use of immunohistochemistry, and others evaluate the amplification of the gene with different techniques, such as: FISH, SISH and real-time PCR. There is also the possibility of using the ELISA (Enzyme Linked Immunoabsorvent Assay) technique to measure the antigen in the serum according to the NCCN guide (2016)

Immunohistochemistry (IHC). (23,24)

IHC is a semi-quantitative technique used for the quantification of protein expression; reveals different epitopes of the protein present on the cell surface, and is the most widely used technique to detect and quantify the HER2/neu protein in the first instance. This technique detects the HER2/neu receptor on the cell membrane using antibodies that bind

to the HER2/neu receptor. This receptor is the target to which the therapeutic agent trastuzumab binds, and therefore the overexpression of this protein should predict the response to this agent. There are many variables that affect the result of immunohistochemistry: fixation, tissue storage, antigen retrieval, type of antibody, measurement system, and interpretation variability among observers. Prolonged fixation in formalin causes changes in protein configuration that lead to masking of antigenic sites and can cause false negatives. The use of 10% buffered formalin with a fixation period of 6 to 12 hours is recommended. It should be taken into account that prolonged storage of paraffin-embedded tissues can cause false negatives, associated with antigen degradation

The interpretation of the results is based on the assessment of the intensity of the staining of the cell membranes and the percentage of positive tumor cells. Results are reported on a scale of 0 to 3 + as follows:

IHC 0.1+: HER2 negative :incomplete membrane staining that is faint/barely perceptible in more than 10% of tumor cell

IHC 2+: equivocal result, weak to moderate complete membrane staining observed in more than 10% of tumor cell in this case FISH tests are performed or a new IHC or FISH test is requested

IHC 3+: HER2 positive: circumferential staining that is complete intense in more than 10% of tumor cell .

Fluorescent in situ hybridization (FISH). FISH is a molecular cytogenetic method that allows the number of copies of a gene to be quantified. FISH is currently considered the gold standard for evaluating HER2/neu amplification, it has a sensitivity and specificity of 98% and 100%, respectively . The currently recommended algorithm for HER2/neu evaluation is as follows:

Initial screening with immunohistochemical test.

All patients with a score of 2+ should be Reffered to FISH, due to the high rate of false positives. Patients with a score of 3 + by immunohistochemistry and those with a score of 2 + who have FISH amplification are considered eligible for treatment with trastuzumab.

Patients with a score of 0 or 1+ and those with a score of 2+ who do not amplify with FISH are considered ineligible (25,26)

Silver in situ hybridization (SISH)

Silver-enhanced in situ hybridization (SISH) has been developed as alternative method to FISH and CISH for HER2 determination .SISH is a novel bright-field in situ hybridization technique similar to CISH. It is a fully automated system developed by (Ventana Medical System) that improves the efficiency and consistency of brigh field in situ hybridization and reducing the risk of error.

Interpretation of results done as follow

Dual Probe ISH Group Definitions:

Group 1 = HER2/CEP17 ratio ≥2.0; ≥4.0 HER2 signals/cell

Group 2 = HER2/CEP17 ratio ≥2.0; <4.0 HER2 signals/cell

Group 3 = HER2/CEP17 ratio <2.0; ≥6.0 HER2 signals/cell

Group 4 = HER2/CEP17 ratio <2.0; ≥4.0 and <6.0 HER2 signals/cell

Group 5 = HER2/CEP17 ratio <2.0; <4.0 HER2 signals/cell

Reporting Results of HER2 Testing by In Situ Hybridization (dual-probe assay)

Result	Criteria(dual-probe assay)
Negative	Group 5
Negative	Group 2 and concurrent IHC 0-1+ or 2+ Group 3 and concurrent IHC 0-1+ Group 4 and concurrent IHC 0-1+ or 2+
Positive	Group 2 and concurrent IHC 3+ Group 3 and concurrent IHC 2+ or 3+ Group 4 and concurrent IHC 3+
Positive	Group 1

2. PATIENTS and METHODS

This was a cross-sectional study conducted during the period from january 2021 to August 2021. Included 50 Iraqi women with proved diagnosed invasive breast cancer with equivocal IHC result for Her2\Neu that where referred to ISH unit for study of gene amplification and the tumor tissues were tested for receptors expression and Her2/Neu

status; automated SISH for consecutive slides from the same paraffin blocks as for Her2/Neu IHC where stained according to manufactures protocol for INFORM Her2 Dual ISH DNA probe cocktail on ventona Benchmark R XT slide stainer.

Staining protocol for HER2 ISH by ventana BenchMark XT automated slide stainer

Deparaffinization	selected
Extended deparaffinization	not selected
Cell conditioning	selected cell conditioning CC2
	mild CC2 8minutes
	standard CC2 12 minutes
	extended CC2 8 minutes
ISH protease3	16 minutes
Denaturation	20 minutes
Hybridization	6 hours
Stringency wash temperature	72Deg C
SISH multimer	16 minutes
Silver chromogen	4 minutes
Red ISH multimer	24 minutes
Red chromogen	8 minutes
Counterstain	Hematoxylin II-8 minutes
Post counterstain	Bluing reagent 4 mi

Statistical analysis:

Data of the 50 patients were entered and managed using Microsoft Excel program version 2016. Analysis of data and extraction of findings and correlations was managed with the statistical package for social sciences (SPSS) software for windows, version 26. Descriptive statistics presented as frequency (No.) and percentages (%) for categories. Also mean and standard deviation was calculated for age of the patients as scale variable. Pie – chart distribution used to demonstrate the distribution of the studied group according to the HER2/neu status. Chi square test used to assess the significance of association between HER2/neu status from one side and each of ER, PR receptor status and age of patient from

the other side. Further analysis was performed using binary regression analysis to control the possible effect of age on the correlation between ER,PR receptors status and HER2/neu status . In all statistical analyses the level of significance of ≤ 0.05 considered significant. Finally, results expressed in tables ,figure and picture with interpretation for each using Microsoft Office Word Program version 2016.

3. RESULTS

A total of 50 patients were enrolled in this study with a mean age of 47.2 ± 9.9 (range: 30 – 75) years, moreover a significant higher proportion of patients (90%) were older than 40 years , (P. value < 0.001), (Table 1). Hormonal status of the studied group revealed that 10 patients (20%) were ER-, PR- and the remaining 40 patients (80%) were ER+, PR+, with significant higher frequent ER+, PR+ status, (P<0.001), (Table 2). Distribution of the studied group according to HER2/neu status using Pie-chart showed not amplified HER2/neu status in 31 (62%) of cases and amplified HER2/neu status in 19 (38%) of cases, indicated significant higher frequency of not amplified HER2/neu status, (P. value = 0.002). Relationship between ER,PR receptors status and HER2/neu status of the studied group is shown in (Table 3). where a significant association was found between ER-, PR- status and amplified HER2/neu status , (P. value = 0.020) on the other hand , calculation of odds ratio indicated that patients with ER-, PR- were about 5.44 folds more likely to have amplified HER2/neu , (Odds ratio = 5.44). Further comparison was performed to assess possible correlation between age and HER2 status , this comparison was statistically insignificant, (P. value > 0.05), (Table 4).

Table1. Age distribution of the studied group

Age (year)	No.	%	P. value
≤ 40	10	20.0	< 0.001
41 - 50	28	56.0	
> 50	12	24.0	
Total	50	100.0	
Mean age ± SD	47.2 ± 9.9		
Range	30 – 75		

SD: standard deviation of mean

Table 2. Hormone receptors status of the studied group

ER,PR receptors status	No.	%	P. value
ER-, PR-	10	20.0	< 0.001
ER+, PR+	40	80.0	
Total	40	100.0	

Table 3. Cross-tabulation for the relationship between hormone receptor status and HER2/neu status of the studied group

ER,PR receptors	HER2/neu status				Total	P. value
	Amplified		Not amplified			
	No.	%	No.	%		
ER-, PR-	7	70.0	3	30.0	10	0.020
ER+, PR+	12	30.0	28	70.0	40	
Total	7	14.0	3	6.0	10	
Odds ratio for ER-, PR- state = 5.44						

Table 4. Cross-tabulation for the relationship between age and HER2/neu status of the studied group

Age (year)	HER2 status				Total	P. value
	Amplified		Not amplified			
	No.	%	No.	%		
≤ 40	4	40.0	6	60.0	10	0.204
41 - 50	13	46.4	15	53.6	28	
> 50	2	16.7	10	83.3	12	
Total	19	38.0	31	62.0	50	

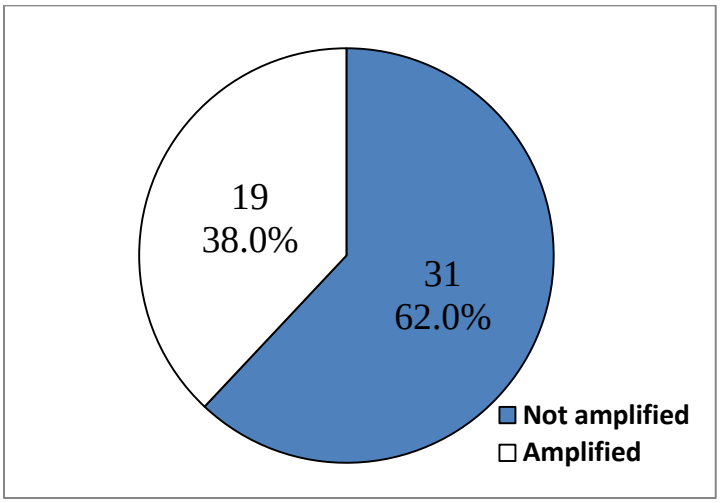

Figure 1. Pie-chart showing the HER2/neu status of the studied group (P. value = 0.002)

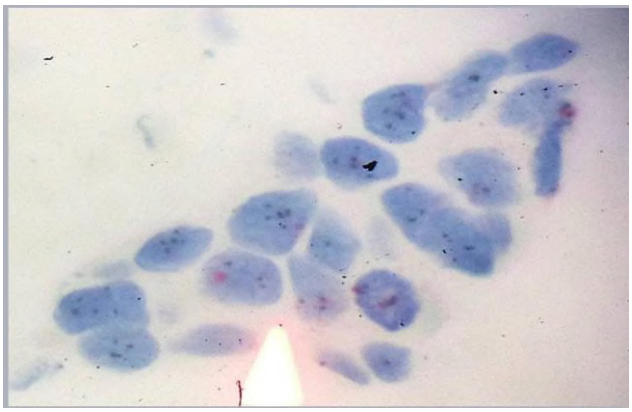

Figure 2. One-Two copies of chromosome 17 red ISH signal present(60x)

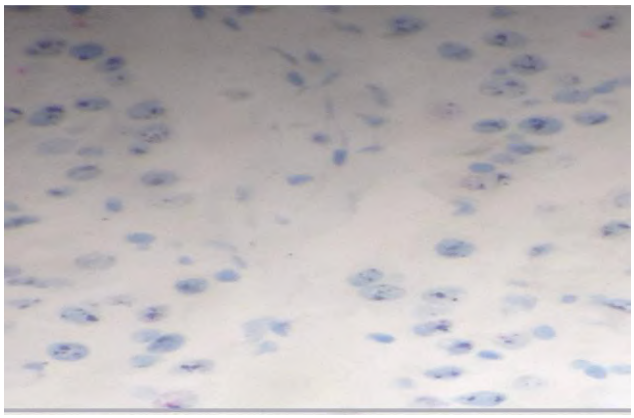

Figure 3. HER2 amplified with presence of HER2 clusters(40x)

4. DISCUSSION

The importance of determining HER2 status lies in the fact that those patients who have breast cancer in advanced stages of the disease and who also present Her2 amplification have greater resistance to conventional chemotherapy and hormonal treatment , in addition to a lower survival rate. The reason why expression of HER2/neu is amplified at 20 to 30 is unknown.This gene is associated with a poor prognosis and survival of the disease; however, currently the monoclonal antibody called Herceptin or trastuzumab inhibits the proliferation of cells in which this oncogene is overexpressed (30–33). Determination of the Her-2/neu gene amplification status is crucial in breast cancer because it allows selecting patients who would benefit from treatment where the treatment agent designed to recognize and bind to HER2 protein and according to published clinical studies, it reduces the risk of early-stage cancer recurrence and reduces the relative risk of death by 20% in patients with metastatic breast cancer (34,35). There are different ways to detect the amplification of the Her-2/neu gene or the increase in the expression of the HER2 protein in the tumor. If you want to detect gene amplification, you can use Southern Blot , fluorescent in situ hybridization (FISH) or PCR; for transcript enhancement Northern Blot , and for HER2 protein enhancement immunohistochemistry (IHC). The technique chosen by Pathology laboratories for being sustainable cost/benefit is IHC. The limitations of this method are reflected in a group of tumors with an undefined 2+ result (intermediate results between positive 3+ and negative 0/1+). In these cases, FISH is considered a gold standard (24,25,27,29) .

The current study aimed to assess Her2/Neu status by Silver insitu hybridization in immunohistochemistry equivocal cases of invasive breast carcinoma, among sample of 50 Iraqi breast cancer patient. The age distribution of the studied group revealed that majority of the patients were older than 40 years, which indicated that the risk of breast cancer is higher in older age women. These finding was not unexpected as the older age is important risk factor of breast cancer where the risk increases significantly with advancing age. Our findings consistent with epidemiological studies regarding the risk factors of breast cancer. Kamińska et al. mentioned that more than 50% of cases diagnosed with breast cancer aged between 50-59 years and the risk increases with the older age (36). According to the American cancer Society , only 4% of diagnosed women with breast cancer are younger

than 40 years (37). In the present study both ER and PR were negative in 20% of the cases while both positive in majority (80%) of cases. These findings agreed that reported in previous studies; Hu et al documented that 80% of breast cancers were hormonal receptor positive (38). On the other hand, heterogeneous, i.e. ER-/PR+, expression of receptor is not common subtype, and PR expression have shown to be not associated with prognosis of ER- breast cancer (39). However, assessment of hormone receptors expression is one of the crucial components of pathological assessment of breast cancer (40). From other point of view, there is a strong evidence about the importance of the biologic, prognostic and predictive role of ER expression in breast cancer, but, the additive role of PR assessment is still controversial (41). Nonetheless, the American Society of Clinical Oncology and American Pathologist College recommended the testing of both ER and PR(39). Regarding HER2/neu status in our study we found that it was not amplified in 32% and amplified in the remaining 38%. Earlier study conducted by Panvichian et al. detected HER2 expression with IHC on formalin fixed paraffin embedded sections of 37 breast carcinoma tissues and found that found that HER2/neu amplified in 29.7% of 37 breast carcinoma(42).

Another study conducted by Murthy et al. found that 73.5% of the IHC 2+ cases were negative for HER2/new amplification, 25% were positive and only 2.2% were equivocal. Thus they concluded that IHC HER2 equivocal patients were heterogenous group and needed FISH for more precise assessment . The heterogeneity between IHC and FISH could be attributed to the polysomy 17 and HER2/neu genetic heterogeneity in equivocal cases (43). Furthermore, Panjwani et al. confirmed that IHC is a wise 1st step to screen tissue samples HER2/neu status to determine about the demand for FISH test as gold standard for assessing HER2/neu status in IHC equivocal breast cancer patients(44).

Also we found that patients with ER-/PR- expression were about 5.4 fold more likely to have amplified HER2/neu , (Odds ratio = 5.44). Moreover, we analyzed the association between ER, PR receptors expression and HER2/neu status after adjustment for age using regression analysis and found that HER2/neu status still significantly associated with ER-, PR- independent of age , (OR=5.13, P. value = 0.035). Our findings consistent with that reported by Huang et al. (45) who studied 1362 breast cancer cases and found that ER, PR were independent. We found no significant association between HER2/neu status and age of the patients, however, previous studies did not found significant association with the age but

Huang et al. found significant inverse association between HER2/neu and age in patients older than 45 years but not in younger group .

5. CONCLUSIONS

As it well documented in previous literature we concluded that frequency of breast cancer increases with advancing age. There is higher proportion of ER+, PR+ status among the studied group. ER, PR and HER2/neu status were significantly correlated and the ER negative ,PR negative status was significantly associated with amplification of HER2/neu independent of patients' age. Silver insitu hybridization is useful in assessment of HER2/ neu status in immunohistochemistry equivocal cases of invasive breast carcinoma. However, we recommended further studies with larger sample size to get further assessment.

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