BREAST CANCER

Use of Immunohistochemistry and Silver in Situ Hybridization (SISH) in Evaluation of Human Epidermal Growth Factor Receptor2 (HER2/Neu) Status in Iraqi Patients with Breast Cancer

Alaa Qasim Yahya[1)], Mohanned Munther Abdul-Ghani[1)], Enass Abdul Kareem Dagher Alsaadi[2)]

ABSTRACT

Background: **Breast cancer is the commonest cause of cancer related death in women worldwide. Amplification or over-expression of the ERBB2 (HER/neu) gene occurs in approximately 15-30% of breast cancer cases and it is strongly associated with an increased disease recurrence and a poor prognosis. Determination of HER2/neu status is crucial in the treatment plan as that positive cases will respond to trastuzumab therapy. It has been used to test for HER2/neu by immunohistochemistry as a first step and then to study only the equivocal positive cases (score 2+) by in situ hybridization technique.**

Aims: **The aim of our study is to compare between immunohistochemistry and silver in situ hybridization (SISH) in assessment of human epidermal growth factor (HER2/neu) receptors status among breast cancer patients**

Methods and Materials: **A cross-sectional study was conducted at The Medical City in Baghdad, from October 2016 to April 2017, where formalin fixed, paraffin embedded tumor tissue from 52 female patients (aged 33 to 71 years) with the diagnosis of breast cancer have been included in this study for the evaluation of human epidermal growth factor (HER2/neu) protein overexpression and gene amplification by immunohistochemistry and silver in situ hybridization (SISH) method respectively. Base line data about patients were obtained from their hospital records.**

Results: **The distribution of human epidermal growth factor (HER2/neu) protein overexpression by immunohistochemistry showed that the (+2) score/equivocally positive were seen in 24 cases, which comprise the largest number of cases in this study (46.15%). Ten cases (19.23%) for each of scores 0/negative and +1/negative were present, while (+3) score/positive was the smallest group with only 8 cases (15.38% of cases). All results of SISH were negative in the category of (0 score/negative). In the contrary, all the 8 cases in (+3 score/positive) category were showing positive SISH results. Category (+1 score/negative) HER2 showed two cases (20%) with a positive SISH result, while that of (+2 score/equivocal positive) showed 8 positive cases (about 33.33%).**

Conclusion: **For the detection of HER2/neu protein overexpression and gene amplification, immunohistochemistry (IHC) and in-situ hybridisation (ISH) are the recommended techniques, with high concordance between the two techniques, however; performance of silver in situ hybridization in assessment of HER2/neu gene status in all cases of invasive breast carcinoma is recommended as significant number of negative cases by immunohistochemistry showed positive amplification by in situ hybridization and can be treated with anti-HER2 targeted treatments.**

KEY WORDS

breast cancer, HER2/ neu, silver in situ hybridization, immunohistochemistry

INTRODUCTION

Breast cancer is the most common cause of cancer related death in women worldwide. Many of the well-established risk factors are those linked to hormones, especially the estrogens. Obesity, early menarche and late menopause are related to increased risk for breast cancer while childbearing and breast feeding reduce the risk, especially with early first pregnancy and increased number of pregnancies[1)]. The incidence rate of breast cancer in Iraq had increased from 26.6 per 100,000 in year 2000 to 31.5 per 100,000 in year 2009, with more cases occurring at younger age groups[2)].

Human Epidermal Growth Factor Receptor 2 (HER2/*neu*)

Human epidermal growth factor receptor *2 (HER2/neu)* is a proto-oncogene; located in long arm of chromosome 17 (17q12). The ERBB family consists of four plasma membrane-bound receptor tyrosine kinases. Other members of this family are human epidermal growth factor receptor, or HER1/*neu*, epidermal growth factor receptor, ERBB -3 (neuregulin-binding), and the ERBB -4. All the four kinases contain an extracellular ligand binding domain, transmembrane domain, and an intracellular domain which can interact with a multitude of signaling molecules and exhibit both a ligand-dependent and a ligand-independent activity. HER2/*neu* can heterodimerise with any of the other three receptors and it is considered to be the preferred dimerisation partner of the other ERBB receptors. Dimerisation results in autophosphorylation

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1) Pathology Department, Al-Kindy College of Medicine
University of Baghdad
Baghdad, Iraq
2) Pathology Department, College of medicine, Karbala University
Karbala, Iraq
Correspondence to: Alaa Qasim Yahya
(e-mail: alaakasim@kmc.uobaghdad.edu.iq)

of tyrosine residues within the cytoplasmic domain of the receptors and will initiate a variety of signaling pathways[3-5], which promotes cell proliferation and stops apoptosis, and therefore must be regulated to prevent uncontrolled cell growth from occurring[6].

Amplification or over-expression of the ERBB2 gene occurs in approximately 15-30% of breast cancer cases and is strongly associated with increased disease recurrence and a poor survival. Over-expression is also known to occur in other tumors like ovarian, gastric, and aggressive forms of endometrial cancer[5,7,8].

Drugs Targeting HER2/neu

HER2/*neu* is a target for the monoclonal antibody therapy, like trastuzumab, which is effective only in cases where HER2/*neu* is over-expressed. An important downstream effect of trastuzumab binding to HER2/*neu* is an increase in p27, a protein halts the cell proliferation. Another monoclonal antibody, the Pertuzumab, inhibits dimerization of HER2 and HER3 receptors, and was approved by the FDA for use in combination with trastuzumab in treatment of breast cancer. Over-expression of HER2/*neu* can also be suppressed by the amplification of other genes. Research is currently being conducted to discover which genes may have this desired effect[9,10].

Experts recommend that every case with invasive breast cancer be tested for the presence of HER2/*neu* amplification because the results will significantly impact treatment recommendations and decisions. For ductal carcinoma in situ, HER2/*neu* testing is not done routinely, except in clinical trials. Retesting for HER2/*neu* whenever breast cancer recurs or spreads must be done, as well as for hormone receptor status, the ER (estrogen receptors), as these can be changed from the original diagnosis[9].

Types of Tests Used to Evaluate HER2/neu Status:

Immunohistochemistry (IHC test): The Immunohistochemistry test finds out if there is HER2/*neu* protein overexpression in the cancer cells. The results of the IHC test can be: 0 (negative), 1+ (negative), 2+ (equivocal), or 3+ (positive) HER2/*neu* protein overexpression[11].

In Situ Hybridization (**ISH test**): The in situ hybridization test finds out if there is HER2/*neu* gene amplification in the cancer cells. The results of the ISH test can be positive (HER2/*neu* gene amplification) or negative (no HER2/*neu* gene amplification)[12].

IHC versus ISH:

In an IHC assay, a slice of tumor tissue is stained, along with a 3+ control specimen, and then examined with a light microscope. The intensity of staining and the percentage of cells stained will correlate with the quantity of HER2 protein[13]. Subjective decisions in scoring are opportunities for variability in an IHC HER2/*neu* assay. With immunohistochemistry, the results can vary based on tissue fixation duration and what antibody had been used for the staining and so many variables can affect the results. The scoring is based on how strong the staining is, which can be quite subjective. Two pathologists looking at the same slide, one might call it 2+/equivocal positive staining and other might call it 3+/ positive staining[14].

Interpreting an ISH HER2/*neu* test, on the other hand, is a much more objective process, where the pathologist counts actual copies of HER2/*neu* genes[15].

However, in daily practice between 80 and 90 percent of primary HER2/neu testing is done with IHC, while only 10 to 20 percent is done with ISH. Approximately 10 percent of IHC test results fall into the so-called "indeterminate" or "equivocal" range and those specimens will re-tested using ISH[16].

Different types of in situ hybridization tests are available with different criteria including florescent in situ hybridization (FISH), chromogenic in situ hybridization (CISH) and silver in situ hybridization technique (SISH).

Silver in Situ Hybridization (SISH)

Using SISH (Silver *in Situ* Hybridization), individual HER2/*neu* genes are clearly visualized and amplification is easily observed, with a control to distinguish it from duplication of the whole chromosome. In addition SISH tests can be archived indefinitely, and require only a standard, bright-field microscope to clearly identify a single gene from the other ~25,000 genes in the cell[17,18]. SISH is similar to FISH, however, it enables bright field detection, a permanent signal, and for simultaneous visualization of underlying tissue morphology, the use of full-strength H&E staining[19]. HER2/*neu* SISH test can be fully automated, requiring six hours run on automated stainer, allowing the lab to do an overnight run, and interpret the test the next day. That six-hour turnaround time compares to the two to three days to do a FISH test[18]. So, the SISH advantages over FISH include faster results and easier to use than the conventional fluorescent in situ hybridization procedure (FISH), and can be read using a standard bright field light microscope with no need for florescent microscope. The permanent signal, which does not fade or bleach, was another big advantage. SISH might be applied to detect any gene or DNA sequence, and therefore could provide superior results in almost any in situ hybridization technique[20].

The aim of this study is to compare between immunohistochemistry (IHC) and silver in situ hybridization (SISH) technique in assessment of human epidermal growth factor (HER2/*neu*) receptors status among breast cancer patients

MATERIALS AND METHODS

A cross-sectional study was conducted in the Medical City in Baghdad, from October 2016 to April 2017, where formalin fixed, paraffin embedded tumor tissue from 52 female patients (aged 33 to 71 years) with the diagnosis of breast cancer have been included in this study for the evaluation of human epidermal growth factor (HER2/*neu*) protein overexpression and gene amplification by immunohistochemistry and silver in situ hybridization (SISH) technique respectively. Baseline data about patients were obtained from their hospital records.

The Immunohistochemical staining procedure:

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

Interpretation of IHC Results[21] :

The results of IHC are interpreted as shown in table 1

The silver in situ hybridization procedure:

Ventana Medical Systems (Ventana) INFORM HER2/*neu* DNA probe is designed as an *in vitro* diagnostic tool to detect the amplification of the HER2/*neu* gene via silver in situ hybridization (SISH) in formalin-fixed, paraffin-embedded breast cancer tissue. The specimens will be stained by using a Ventana automated slide stainer, after that examined by using light microscope.

Principle of the Procedure

During the silver in situ hybridization (SISH) staining process, DNP labeled probes are bound to specific target DNA sequences in cells or tissues. The DNP labeled probe is then visualized using Rabbit anti-DNP primary antibody and an ultra-view SISH detection kit. The detection kit contains goat anti-rabbit antibody conjugated to horseradish peroxidase (HRP) that is utilized as a chromogenic enzyme. The chemistry of the SISH reaction is driven by sequential addition of silver A (silver acetate), silver B (hydroquinone) and silver C (H2O2). The silver ions (Ag+) will be reduced by hydroquinone to metallic silver atoms (AgO) and this reaction is fueled by the substrate for HRP, hydrogen peroxide (Silver C). Silver precipitation will be deposited in the nuclei and a single copy of the HER2/neu gene is visualized as a black dot. The specimen is then counterstained for interpretation under the light microscope. Each step in staining protocol includes incubation for particular time at specific temperature and at the end of each incubation step, the sections will be rinsed by Ventana automated slide stainer to stop the reaction and remove the unbound material that would impede the desired reaction in the subsequent step. One to ten ml dispenser of INFORM HER2/

Table 1: HER-2/neu scoring system and criteria for scoring system Criteria

0	Negative. No staining is observed, or membrane staining in < 10% of the tumor cells.
1+	Negative. Incomplete membranous staining is detected in >10% of the tumor cells.
2+	Equivocal positive. Complete membrane staining is observed in > 10% of the tumor cells, with weak to moderate intensity.
3+	Positive. Complete membrane staining is observed in >10% of the tumor cells, with strong intensity.

Table 3: HER2/*neu* protein overexpression by IHC

HER2/*neu* score value	No. of cases
0	10 (19.23%)
+1	10 (19.23%)
+2	24 (46.15%)
+3	8 (15.38%)
Total	52 (100%)

neu DNA probe contain approximately 10 mg/ml of the HER2/*neu* probe labeled with Dinitro-Phenol (DNP), formulated with a human placental blocking DNA in a formamide based hybridization buffer. Determination of HER2/*neu* gene status (ratio of HER2/*neu* to chromosome 17) requires use of Ventana INFORM chromosome 17 probe on a separate slide for each specimen

Interpretation of SISH Results:

Cell Selection Criteria: Only nuclei with diameters that are representative of the average population of invasive carcinoma nuclei should be counted in the targeted area. Signals in nuclei that are much larger or much smaller in diameter than the average size of carcinoma nuclei should be neglected. In targeted areas which are genetically heterogeneous for HER2/*neu* copy number, only nuclei representative of the population of invasive carcinoma nuclei with the highest average number of signals will be counted.

Determining HER2 Gene Status: In SISH, the HER2/*neu* gene appear as black signal and Chr.17 as red signal. Using serial stained slides which meet the criteria for slide adequacy, the number of signals in 20 nuclei will be counted in the same targeted area for each of HER2/*neu* and Chr17. The HER2/*neu* to Chr17 ratio will be calculated by dividing the total number of HER2/*neu* signals by the total number of Chr17 signals. Cases with a HER2/Chr17 ratio less than 1.8 are negative for HER2 gene amplification while cases with a HER2/Chr17 ratio more than 2.2 are positive for HER2 gene amplification[22-24]. For cases with a HER2/*neu* to Chr17 ratio between 1.8 and 2.2, an additional targeted area will be selected for each of HER2/*neu* and Chr17 and will count the number of signals in 20 nuclei and calculate the HER2/*neu* to Chr17 ratio. Cases with a HER2/*neu* to Chr17 ratio less than 1.8 are negative for HER2/*neu* gene amplification while cases with a HER2/Chr17 ratio more than 2.2 are positive for HER2 gene amplification. Cases with a HER2/*neu* to Chr17 ratio again between 1.8 and 2.2 are considered equivocal for HER2 gene amplification[22,25].

Controls: To ascertain that all reagents are functioning properly, stained positive reagent and control specimen should be examined first. The presence of silver depositions within the cell nuclei is indicative of positive reactivity. Normal cells in the field of view will serve as an internal control of the staining run. Normal cell nuclei should contain on average 1-2 discrete dots, indicating that the HER2/*neu* DNA probe has hybridized to the gene. Failure to detect the single gene copy in normal cells indicates the run failure and the results would be considered invalid.

Statistical Analyses

Statistical analysis was done using statistical package for social sciences version 18 (SPSS V.18, Chicago, IL, USA). Chi square test was used to test the association between discrete variables. Unpaired T test was used to find the means of normally continuous samples of two groups of data. All P values used were asymptotic and two sided. Findings with P value less than 0.05 were considered significant.

Table 2: Age group distribution of the study cases

Age group	No. of cases	Percentage	Mean class interval
30-39 years	18	34.61%	
40-49 years	18	34.61%	
50-59 years	4	7.69%	46.8 years old
60-69 years	10	19.23%	
≥ 70 years	2	3.84%	
Total	52	100%	

Table 4: Percentage of positive cases for HER2/neu gene amplification by SISH within each score

HER2/*neu* value	No. of positive SISH cases	No. of negative SISH cases
0	0(0%)	10 (100%)
+1	2 (20%)	8 (80%)
+2	8 (33.33%)	16 (66.66%)
+3	8 (100%)	0 (0%)
Total	18 (34.61%)	34 (65.38%)

RESULTS

Among the 52 cases of breast cancer enrolled in this study, 42 (80.76%) of the cases showed histological features of invasive ductal carcinoma while 8 (15.38%) of cases, showed invasive lobular carcinoma and two (3.84%) of cases were of mixed type.

Age distribution showed that the higher rates of breast cancer between ages 30-49 years (69.22%) followed by age group 60-69 years that comprises 19.23% of cases, while the age groups 50-59 and the ≥ 70 years where comprising about 7.69% and 3.84% respectively. Median age of the study patients was 46.8 years old as shown in table 2.

Involvement of one breast (unilateral breast carcinoma) occurs in 34 (65.38%) of the current study cases, while involvement of both breasts (bilateral breast carcinoma) occurs in 18 (34.61%) of these cases.

Distribution of HER2/*neu* score value by IHC showed that the score (+2/equivocal positive) were seen in 24 (46.15%) of the cases, which comprises the highest percentage of the cases among current study. 10 (19.23%) of the cases for score (0/negative) and 10 (19.23%) of the cases for score (+1/negative). Score (+3/positive) was smallest number of cases with 8 (15.3.38%) of the cases as shown in table 3.

All results of SISH were negative in category of sore 0 (0%) which consist of 10 cases in which no amplification of HER2/*neu* gene was seen.

In the contrary, all the 8 cases (100%) of score +3 HER2/*neu* category were showing positive SISH results, i.e. show amplification of HER2/*neu* gene.

Category +1 HER2/*neu* showed two cases (20%) with a positive SISH result, while the remaining 8 cases (80%) show no amplification of HER2/*neu* gene.

Category +2 HER2/*neu* showed 8 positive (33.33%) cases that show amplification of HER2/*neu* gene and the remaining 16 (66.66%) cases show no amplification as shown in table 4.

DISCUSSION

Breast cancer is one of the most common malignancies in females and detection of HER2/*neu* gene status in them is very crucial to determine the mode of treatment[1,3].

In current study, 52 cases of invasive breast cancer were studied for HER2/*neu* protein overexpression and gene amplification with immunohistochemistry and SISH respectively.

By immunohistochemistry testing, the tissue specimens were stained and the reviewer interprets the resulting intensity and uniformity of membrane staining quantitatively and qualitatively on a scale from 0 to

+3[21]. While all of the 8 cases with HER2/*neu* score +3 where showing amplification of the gene using the SISH method, only 8 (i.e. 33.33%) of the 12 cases with HER2/*neu* score +2 show amplification of the gene. This is in agreement with what Ramier *et al*[26] in Italy have found in their study, 28% of score +2 showed positive gene amplification using the SISH method. While Bae *et al* in Korea found that 62.2% of score +2 showed gene amplification by SISH[27].

According to the American Society of Clinical Oncology and the College of American Pathologists (ASCO/CAP) joint HER2/*neu* Guidelines Recommendations, a score of (0) or (+1) is considered a negative test result, (+2) is equivocal, and (+3) is considered positive for HER2[15]. An updated HER2-testing algorithm has been proposed to include bright-field ISH for the retesting of specimens with equivocal (+2) immunohistochemical scores[21]. In current study, two negative cases with score (+1) HER2/*neu* showed positive SISH results on Ventana digital imaging , comprising 20% of score 1+ and 10% of the total negative cases. Lower than this percentage was found by Bae *et al* who found that 13 (1.7%) cases out of 756 cases with negative IHC results showed gene amplification by SISH[27].

In this study, all the 8 (100%) positive cases were also positive for gene amplification by SISH in agreement with Bae et al who found 99.4% of score +3 had HER2/neu gene amplification by SISH[27].

Because HER2/*neu* gene amplification is directly linked to protein expression levels in breast cancers, IHC for HER2/*neu* protein expression and ISH for HER2/*neu* gene amplification can replace each other. DNA is usually less affected by the tissue processing artifacts, and experimental errors occur with less frequency during HER2/*neu* gene assessment by ISH. IHC results can be affected by many critical factors like the tissue processing (preanalytic factors), the antigen retrieval methods, the type of reagents and primary antibodies used (analytic factors), and the subjective interpretation of the staining results (postanalytic factors)[28].

The incidence of HER2/*neu* positive tumors among primary breast carcinomas is expected to be 18% to 20%[27]. Among the 52 samples in this study, HER2/*neu* protein overexpression was observed in 15.38% of samples (score+3) with 46.15% of the cases showed equivocal results (score+2), and HER2/*neu* gene amplification by SISH analysis was observed in 18 cases (34.61%) of samples. Among 959 samples in Bae *et al* study, HER2/*neu* overexpression was observed in (17.3%) of samples, and HER2 amplification by SISH analysis was observed in (21%) of samples[27]. A similar incidence of HER2/*neu* positive tumors was also observed in recently published Korean study performed in a single institution in which Park *et al*[29] reported HER2/*neu* protein overexpression and HER2/*neu* gene amplification by FISH (florescent in situ hybridization) in 16.8% and 24.2% of 950 Korean patients with invasive breast cancers, respectively. Mohammed Ali *et al* in their study performed on samples from 448 Iraqi patients with invasive breast carcinoma found that 144 (32%) of the cases showed positive results by CISH (chromogenic in situ hybridization) for HER2/*neu* gene amplification and they recommended to use in situ hybridization for all cases of invasive breast carcinoma as that 20% of score +1/negative cases showed gene amplification by CISH[30].

CONCLUSIONS

Although the current recommendations for HER2/*neu* testing are either for a two-tier system using IHC with reflex ISH testing for equivocal positive cases, or as a one-tier ISH strategy, it may be more useful to start with in situ hybridization test for HER2/*neu* as many cases reported as negative by immunohistochemistry showed gene amplification by in situ hybridization and that measurements of HER2/*neu* gene amplification by SISH are less affected by the preanalytic factors than measuring HER2/*neu* protein overexpression by IHC.

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