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Evaluation of Immunohistochemistry-Equivocal (2+) HER2 Gene Status in Invasive Breast Cancer by Silver DNA in Situ Hybridization (SISH) and its Association with Clinicopathological Variables

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KEYWORDS	ABSTRACT
HER2 SISH Breast carcinoma Immunohistochemistry Equivocal	Background and Objective: Determination of HER2 gene is crucial in breast carcinoma management and prognosis, as HER2 alterations are linked to a shorter disease-free period, overall survival and resistance to tamoxifen anti-estrogen therapy and other chemotherapy regimens, regardless of the nodal or hormone receptor status. This study aimed to estimate HER2 gene status of infiltrative mammary cancer cases with immunohistochemically equivocal (2+) score using Silver DNA in Situ Hybridization (SISH) technique and to investigate its association with clinicopathological variables. <i>Methods:</i> The study included 52 formalin-fixed paraffin embedded tissue blocks from female patients with invasive breast carcinoma with score of 2+ (equivocal) HER2 immunohistochemistry. All cases were studied by silver DNA in situ hybridization technique (SISH) for the determination of the amplified HER2 DNA. <i>Results:</i> The SISH technique showed that HER2 gene was not amplified in 33 cases out of 52 (63.5%); while the rest of 19 cases (36.5%) revealed amplified gene status. According to age, HER2 gene status reported non-significant difference in the age groups between cases with amplified and non-amplified gene status (<i>P</i> =0.173). There was a significant negative association between positive Estrogen (ER) and
Article Info	Progesterone (PR) status and HER2 gene amplification (P = 0.002 and 0.017, respectively).
	Conclusion: More than half of breast carcinoma cases with equivocal HER2
Received 12 Oct 2015;	immunoreactivity showed non-amplified gene status; this needs to be considered by
Accepted 11 Apr 2016;	oncologists in their management planning of breast cancer. Amplified HER2 gene is
Published Online 30 Aug 2016;	significantly associated with negative ER and PR status that affects patients' management protocols and future outcome of the disease.
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Introduction

Breast cancer is one of the most common causes of death, worldwide. The increasing incidence of breast cancer and its associated mortality highlight the need for new therapeutic development, especially targeted treatment (1).

The course of breast cancer is influenced by several prognostic parameters, including

histological type and grade, tumor size, proliferation index, lymph node status, lymphatic and vascular invasion, Estrogen (ER) and Progesterone (PR) receptor status etc. (2).

Amplification of HER2 gene is associated with rapid progression of the disease, increased metastatic potential and resistance to tamoxifen. The discovery of targeted therapy against the HER2 gene (Herceptin) has brought an effective treatment modality for breast cancer patients (3).

Immunohistochemistry (IHC) is the most frequently used, convenient and cost-effective initial test for HER2 protein expression. HER2 IHC results are generally divided to four scale scores (range, 0 to 3+), depending on the percentage of positive tumor cells and staining intensity. The US Food and Drug Administration (FDA) recommends that HER2 IHC scores of 0 and 1+ must be considered as HER2 negative and those with HER2 (3+) scores should be regarded as HER2 positive while HER2 equivocal invasive breast cancer are those with HER2 (2+) score. This has to be further assessed by Fluorescent In Situ Hybridization (FISH), which is more accurate and reliable than IHC. However, the need for a skilled operator, long procedure, special equipment, and difficult preservation of slides for later review are the disadvantages (4).

A fully automated method, Silver In Situ Hybridization (SISH) may overcome the disadvantages of FISH and it has the same accuracy of FISH. It is performed by counting black signals, which are dot-like on the conventional bright field light microscopy (5). "Advantages of SISH include high sensitivity for detection of single gene copies, quantifying DNA targets with high resolution, and tissue counterstaining with high contrast for visualizing the signal separately and determining tissue morphology" (6).

The aim of the present work was to estimate HER2 gene status of infiltrative breast cancer cases with immunohistochemically equivocal (2+) score using the SISH technique and to investigate its association with some clinicopathological variables including age of patients, type of breast cancer, and ER and PR status.

Materials and Methods

Fifty-two formalin-fixed paraffin embedded tissue blocks from female patients with invasive breast carcinoma with score 2+ (equivocal) HER2 immunohistochemistry were collected during the period from September 2013 to October 2015. All cases were studied by Silver DNA In Situ Hybridization technique (SISH) in a private pathology laboratory to determine the HER2 gene amplification status; amplified versus nonamplified.

All cases were already studied by the immunohistochemistry technique in the same laboratory for ER and PR status; positive versus negative.

Correlation of HER2 gene status by the SISH technique was made with different parameters including: age of the patient, type of invasive mammary carcinoma, and ER and PR immunoreactivity.

Other clinicopathological information including tumor size, stage and lymph node status were not available, because the specimens included in the present work were incisional biopsies. All of the cases were moderately differentiated.

Immunohistochemistry

Immunoreactivity for all cases for HER2, ER, and PR was studied by the VENTANA-BenchMark-XT computerized automated system, using the *ultra*View Universal DAB Detection Kit. Four-micrometer-thickness tissue sections were used.

The antibodies used were:

1- HER2 rabbit monoclonal antibody (clone 4B5).

2- Estrogen receptor rabbit monoclonal antibody (clone SP1).

3- Progesterone receptor rabbit monoclonal antibody (clone 1E2).

The *ultra*View Universal DAB Detection Kit detects specific mouse and rabbit primary antibodies bound to an antigen in paraffin-embedded tissue sections. The specific antibody is located by a cocktail of enzyme labeled secondary antibodies (HRP Multimer). The complex is then visualized with hydrogen peroxide substrate and 3, 3'-diaminobenzidine tetrahydrochloride (DAB) chromogen, which produces a brown precipitate that is readily observed by light microscopy. The principal steps of the procedure is illustrated in Figure 1.

The staining protocols followed for all three immuno-stains (HER2, ER, and PR) were in accordance with standard staining protocols of VENTANA- BenchMark-XT computerized automated system for each antibody. The immunohistochemistry results and scores were recorded independently by two pathologists, followed by a common review for agreement.

The immunoreactivity criterion for inclusion of all the cases in the study (HER2 score 2+, equivocal) was weak-moderate complete circumferential membranous staining in >10% of invasive cancerous cells.



Fig. 1. UltraView Universal DAB Detection Kit Reaction.

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The immunoreactivity criteria for evaluation of all the cases in the study for ER and PR were in harmony with Allred method. Allred scoring system is a combination of proportion score and intensity score. Proportion score is a scale of 0 to 5 (0: no reactivity, 1: <1% of tumor cells, 2: 1-10% of tumor cells, 3: 1/3 of tumor cells, 4: 2/3 of tumor cells, 5: >2/3 of tumor cells). Intensity score is a scale of 0 to 3 (0: none, 1: weak staining, 2: moderate staining, 3: strong staining).

The Allred final score value will fall in a range of 0 to 8; score of 0 to 2 was considered a negative result, and score of 3 to 8 was considered a positive result.

Silver DNA In Situ Hybridization (SISH)

Assessment of HER2 amplification using SISH procedure was studied by the VENTANA-BenchMark-XT computerized automated system; 4 µm thickness tissue sections were used. In brief, the procedure combines two basic detection kits, HER2 & chromosome 17 DNA probes cocktail, and other supplementary bulk solutions and reagents:

1- INFORM HER2 Dual ISH DNA Probe Cocktail: contains a cocktail of HER2 DNA probe and chromosome 17 DNA probe.

2- *ultra*View SISH DNP (dinitrophenyl) Detection Kit: detects the HER2 DNA probe sequence.

3- *ultra*View Red ISH DIG (digoxigenin) Detection Kit: detects chromosome 17 DNA probe sequence.

Each detection kit uses: a chemical compound that labels the DNA probes, primary antibody, secondary antibody (HRP Multimer), enzymes, and a series of chromogen reagents. The principal steps of the procedure for both detection kits are illustrated in Figures 2 and 3.



Fig. 2. Silver DNA In Situ Hybridization Reactions for HER2 DNA Detection

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Fig. 3. Red In Situ Hybridization Reactions for Chromosome 17 DNA Detection

During the staining process, the DNP and DIG labeled probes are co-hybridized to their respective specific target DNA sequences (HER2 and chromosome 17, respectively) within the cell nuclei. Then detection of HER2 signals occurs first by *ultra*View SISH DNP Detection Kit, as a black intranuclear signal, followed by detection of chromosome 17 signals by *ultra*View Red ISH DIG Detection Kit, as a red intranuclear signal.

The staining protocol was in accordance with standard dual ISH staining protocol of VENTANA-BenchMark-XT computerized automated system, the procedure roughly takes 13 hours to complete, it is briefly summarized in Table 1.

The slides were independently reviewed by two pathologists, followed by a common review for agreement. The HER2 black signals and chromosome 17 red signals were enumerated in tumor invasive cells nuclei, according to VENTANA HER2 SISH interpretation algorithm, and results were reported as a function of (HER2/chromosome 17) signals ratio to determine HER2 amplification status: HER2/chr17 ratio of ≥ 2.0 was considered amplified, while a ratio of < 2.0was considered non-amplified.

Statistical analysis

The statistical analysis of this study was performed using SPSS 21.0 (Chicago, IL, USA) and Microsoft Excel 2013. Categorical data were formulated as counts and percentages. Chi-square and Fisher exact tests were used to describe the association of these data. The lower level of accepted statistical significant difference was below or equal to 0.05.

Table. 1. The Staining Protocol in Accordance WithStandard Dual In Situ Hybridization Staining Protocol ofVENTANA- BenchMark-XT Computerized AutomatedSystem

Steps	Times/Note
Deparaffinization	Automated
Cell conditioning	28 minutes
SISH protease	16 minutes
Cell denaturation	20 minutes
Hybridization	6 hours
Stringency wash	Automated, 72 degree centigrade
SISH multimer	16 minutes
Silver chromogen	4 minutes
Red ISH multimer	24 minutes
Red chromogen	8 minutes
Hematoxylin counter stain	8 minutes
Bluing reagent Post counter stain	4 minutes

Results

Clinicopathological parameters

The study included 52 female patients with invasive breast carcinomas. Their ages ranged from 31 to 81 years with 25 cases (48.1%) younger than 50 years and 27 cases (51.9%) aged 50 or older (Table 2).

Concerning the types of invasive breast carcinoma that are included in the present work; the majority of cases (44 out of 52) were of invasive Ductal Carcinoma Not Otherwise Specified (IDC-NOS), comprising 84.6%. The remaining cases were: Invasive Lobular Carcinoma (ILC) (2 cases, 3.8%), mixed IDC-NOS and ILC (2 cases, 3.8%), medullary carcinoma (2 cases, 3.8%), ILC pleomorphic type (1 case, 1.9%) and invasive mucinous carcinoma (1 case, 1.9%) (Table 2).

Immunohistochemical study of the cases revealed that half of the cases were ER positive (26 cases, 50%) (Figure 4) and the other half showed negative ER status, while cases with positive PR status were 27 (51.9%), and other cases showed negative PR immunoreactivity (25 case, 48.1%) (Table 2).



Fig. 4. Invasive Ductal Carcinoma of Breast with Positive Brown Nuclear Staining (arrows) of Estrogen Receptor (ER) Immunohistochemistry of Score 8/8 According to Allred Scoring Method (20X).



Fig. 5. Invasive Ductal Carcinoma of Breast with HER2 Immunohistochemistry Score 2+ (equivocal) Showing Weak-Moderate Complete Circumferential Brown Membranous Staining (arrows) in >10% of Invasive Cancerous Cells (60X).

Table. 2. Descriptive Analysis of the Clinicopathological Parameters of the Studied Cases

Clinicopathological pa	arameters	Count	Percentage
	< 50 yr	25	48.1
Age groups	>=50 yr	27	51.9
	Total	52	100.0
	Negative	26	50.0
ER	Positive	26	50.0
	Total	52	100.0
	Negative	27	51.9
PR	Positive	25	48.1
	Total	52	100.0
	IDC	44	84.6
	ILC	2	3.8
	ILC pleomorphic	1	1.9
Type of cancer	Invasive mucinous CA	1	1.9
	Medullary CA	2	3.8
	Mixed IDC and ILC	2	3.8
	Total	52	100.0

ER: Estrogen Receptor, PR: Progesterone Receptor, IDC: Invasive Ductal Carcinoma, ILC: Invasive Lobular Carcinoma, CA: Carcinoma



Fig. 6. Invasive Ductal Carcinoma With Non-Amplified HER2 Gene by the Silver DNA in Situ Hybridization Technique; HER2 gene black SISH intranuclear signals (arrows) typically appear smaller than chromosome 17 red ISH intranuclear signals (arrow heads) with an overall ratio of (black SISH signals/red ISH signals) < 2 (60X).



Fig. 7. Invasive Ductal Carcinoma With Non-Amplified HER2 Gene by Silver DNA in Situ Hybridization (same case in Figure 6 with higher magnification); each carcinoma cell nucleus shows (on average) an equal number of HER2 gene black SISH signals (arrows) and chromosome 17 red ISH signals (arrow heads) with a ratio of < 2 (100X). 14 Evaluation of Immunohistochemistry-Equivocal (2+) HER2 Gene ...



Fig. 8. Invasive Ductal Carcinoma With Amplified HER2 Gene by Silver DNA in Situ Hybridization; HER2 gene black SISH intranuclear signals (arrows) typically appear smaller than chromosome 17 red ISH intranuclear signals (arrow heads) with an overall ratio of (black SISH signals/red ISH signals) > 2. (60X).



Fig. 9. Invasive Ductal Carcinoma With Amplified HER2 Gene by Silver DNA in Situ Hybridization (same case in Figure 8 with higher magnification): each carcinoma cell nucleus shows an average of 3 to 4 HER2 gene black SISH signals (arrows) and 1to 2 chromosome 17 red ISH signals (arrow heads) with a ratio of > 2(100X)

Table.	3.	Association	Between	Age	and	HER2	Gene	Status
ranc.	J.	Association	Detween	ngu a	anu	IILK2	OCIIC	Status

Age groups		HER2 gene status	Total	
		Amplified	Non-amplified	
< 50 years	Count	7	18	25
	Percentage	36.8%	54.5%	48.1
>= 50 years	Count	12	15	27
	Percentage	63.2%	Non-amplified Non-amplified 18 25 8% 54.5% 48.1 15 27 2% 45.5% 51.9 33 52 0.0% 100.0% 100.0	
Total	Count	19	33	52
	Percentage	100.0%	100.0%	100.0
p- value		0.173NS		
NS: non significant				

NS: non-significant

Table. 4. Association between HER2 Gene Status by Silver DNA in Situ Hybridization and Hormone Receptors Immunoreactivity

			HER2 gene			
Hormone receptor			Amplified	Non - amplified	Total	P- value
	Nagativa	Count	15	11	26	
ED	Negative	Percentage	78.9%	33.3%	50.0	0.002*
EK	Desitive	Count	4	22	26	0.002*
	Positive	Percentage	21.1%	66.7%	50.0	
Tetal		Count	19	33	52	
Total		Percentage	100.0%	100.0%	100.0	
	Negative	Count	14	13	27	
DD		Percentage	73.7%	39.4%	51.9	0.017*
PK -	Positive	Count	5	20	25	0.017*
		Percentage	26.3%	60.6%	48.1	
Tatal		Count	19	33	52	
Total		Percentage	100.0%	100.0%	100.0	

ER: estrogen receptor, PR: progesterone receptor, *: statistically significant difference

Type of invasive breast carcinoma		HER2 gene stat	T-4-1	
		Amplified	Non -amplified	Total
IDC	Count	15	29	44
IDC	Percentage	78.9	87.9	84.6
пс	Count	0	2	2
ILC	Percentage	0.0	6.1	3.8
H C - L L -	Count	1	0	1
ILC pleomorphic	Percentage	5.3	0.0	1.9
L	Count	0	1	1
Invasive mucinous CA	Percentage	0.0	3.0	1.9
Mada la la como CA	Count	2	0	2
Medullary CA	Percentage	10.5	0.0	3.8
	Count	1	1	2
Mixed IDC and ILC	Percentage	5.3	3.0	3.8
T	Count	19	33	52
1 otai	Percentage	100.0	100.0	100.0
P- value		0.206NS		

 Table. 5. Association of HER2 Gene Status by Silver DNA in Situ Hybridization with Types of Invasive Breast

 Carcinoma

IDC: invasive ductal carcinoma, ILC: invasive lobular carcinoma, CA: carcinoma, NS: non-significant

According to age, HER2 gene status showed non-significant difference in age groups between cases with amplified and non-amplified gene status (P=0.173) (Table 3).

The comparison of HER2 gene amplification according to hormone status revealed a significant negative association between positive ER and PR status and HER2 gene amplification by SISH in the studied cases (P= 0.002 and 0.017, respectively) (Table 4), however, the gene amplification status was not significantly different among types of invasive breast carcinoma (P=0.206) (Table 5).

Discussion

Precise evaluation of HER2 status is vital to optimize the outcome of patients with mammary carcinoma (7).

Immunohistochemistry is sufficient for the initial HER2 assessment, but patients with tumors scored less than 3+ (particularly those interpreted as 2+) would benefit from FISH to assess HER2 status more precisely and to avoid erroneous prognostication and inappropriate management (8). Furthermore, FISH is a practically objective and quantitative method in detecting HER2 gene amplification on the nuclei of tumor cells. The drawbacks of this procedure are its high costs (10 times that of IHC), complexity, the need for a

fluorescence microscope, the temporary signal (it requires a special camera), and its inability to perceive and evaluate tumor histomorphology (9).



Fig. 10. Invasive Ductal Carcinoma With Huge Amplification of HER2 Gene by Silver DNA in Situ Hybridization Showing Gene Cluster (signal clusters) Formations; the HER2 gene black SISH clusters appear as grape-like formations (arrows) composed of dozens of individual signals. In such cases with huge amplification, the HER2 gene clusters may mask some of the chromosome 17 red ISH signals. Ratio of (black SISH signals/red ISH signals) = 8.5 (60X)

Technologies for HER2 testing include Chromogenic In Situ Hybridization (CISH) and SISH (10). Numerous earlier works have enrolled cases with HER-2 overexpression (IHC 3+) or HER2 amplification, and reported their correlation with high tumor cell grade, absence of ER or PR expression, DNA aneuploidy, and high Ki67 (11-13).

The current study focused on invasive breast carcinoma cases with equivocal HER2 status by immunohistochemistry (score of 2+) further evaluated by SISH, and the relationship between HER2 gene amplification status and various clinicopathological variables including (age, tumor type, ER and PR status) were assessed. Out of 52 cases of 2+ score of HER2 by IHC, 33 (63.5%) showed nonamplified HER2 gene; and (19 cases, 36.5%) revealed amplified gene status; the amplified gene status was negatively correlated with ER and PR positive expression. There was no significant association between HER2 gene amplification and age of patients or type of invasive breast cancer.

To the best of our knowledge, very few published work are concerned with such group of patients (with equivocal IHC results of HER2) (4, 14-16). Lee et al. (14) considered a large number of cases with invasive breast cancer, among which 419 out of 1735 (24%) were of score 2+ HER2 by IHC. They reported that 14% (57/413) were HER2 amplified, according to FDA criteria (ratio of HER2 signals to chromosome 17 signals ≥ 2.0). Furthermore, HER2 amplification was related to the percentage of complete membrane staining. Chibon et al. (15) studied 108 breast cancer cases with score 2+ HER2 by IHC. Rate of HER2 amplification by FISH was 33%. Tumor grade and percentage of cells showing membrane staining were indicators of HER2 status. Dieci et al. (16) investigated 480 breast cancer tissues with 2+ HER2, revealing that high tumor grade and high Ki67 were significantly associated with HER2 FISH amplification. Payandeh et al. (17) recruited females with breast cancer at the Oncology Clinic, Kermanshah, Iran. Of the 133 patients, 37 were classified as IHC 2+ and 96 were classified as IHC 3+. HER2 was found to be amplified by FISH in 12 out of 37 (32.4%) of the IHC 2+ cases while 25 (67.6%) of the IHC 2+ cases had HER2 FISH negative test results.

The HER2 gene was amplified (positive) in 34.6% (63/182) by FISH, according to FDA criteria, and tumors with HER2 amplification were more

likely to harbor ER negative or PR negative results (4). Wang et al. (18) conducted a recent work on 1036 females with breast cancers. The expression of hormone receptors and HER2 was studied by IHC, while FISH was used to determine HER2 status in cases scored IHC 2+. They suggested that the negative association between ER, PR and HER2 was age-related and only applied for females aged >40 years.

There was a converse association between the expression of ER, PR expression and HER2 amplification in both preclinical and clinical studies (19-23). This inverse relationship has been connected with the fact that estrogen and its receptor are requisites to suppress HER-2/neu. ER and HER-2/neu signaling are inversely correlated throughout a transcriptional repression of HER-2/neu by estradiol binding to ER (24).

Hormone receptors assessment can predict the effectiveness of hormonal therapy in mammary carcinomas (25). HER2-positive tumors have a higher association with ER and PR negativity (1, 26, 27). In positive ER cases, HER2 overexpression was indicated. Such cases have worse survival compared to those showing positive ER and negative HER2 tumors, signifying that HER2 status is a valuable interpreter of response to hormone therapy (28). Expression of PR influences the possibility of HER2 amplification in cases with positive ER (21). A study concluded that HER2 amplification/overexpression was associated with lower ER/PR levels (19).

Recently, a few articles have been published regarding outstanding effectiveness of the HER2 SISH method (29, 30). Concordance between FISH and SISH was 98.2% (31).

Furthermore, FISH is considered a typical method for assessing of HER2 gene (32). However, disadvantages of this method include sophisticated analysis, fast fading of fluorescence, and lengthy procedure (31).

Substitute techniques have been attempted (33). The CISH is a simple satisfactory method to recognize HER2 gene copies using steps similar to immunohistochemistry, ordinary light microscope and everlasting signal intensity (34, 35). Nevertheless, it is manually operated and timeconsuming. Silver DNA in Situ Hybridization is automated and time efficient in addition to the use of ordinary light microscopy and permanent signal intensity (31). Around 12 to 13 hours is required to complete the SISH test, a time that is roughly half that of FISH and CISH, which require an average of 24 hours.

Conclusion

More than half of breast carcinoma cases with equivocal HER2 immunoreactivity showed nonamplified gene status; this result is important to be taken into consideration by oncologists during their management planning of patients with breast cancer. Amplified HER2 gene is significantly associated with negative ER and PR status that affects patients' management protocols and future outcome of the disease.

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