Comparison of Immunohistochemical Methods (IHC) and Fluorescent in Situ Hybridization (FISH) in the Detection of HER 2/Neu Gene in Kurdish Patients with Breast Cancer in Western Iran

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ABSTRACT
Background: Amplification of HER2 is an important factor in the diagnosis and treatment of breast cancer. Fluorescence in situ hybridization (FISH) is the gold standard for the detection of HER2-positive tumors. However, the Immunohistochemistry (IHC) assay for the detection of HER2 is more popular in the preclinical laboratory since it is faster and more economical compared to the FISH test.
Materials and Methods: In this study, the status of HER2 amplification is determined by the FISH test using 44 formalin-fixed paraffin-embedded tissue samples and comparing the results with the IHC test to determine the reliability of the IHC test. Also, the relationship between HER2 amplification and estrogen, progesterone receptors, P53, age, menopausal status, family history of breast cancer, tumor size, and histological grade were determined.
Results: Examination of HER2 in 44 samples by IHC showed 3 (6.8%) and 5 (11.4%) samples were positive (IHC 3+) and negative (IHC 0, 1+), respectively, and 36 (81.8%) samples were ambiguous (IHC 2 +), but examination by FISH showed 21 samples (47, 7%) were positive and 23 samples (52, 3%) were negative. There was a significant difference between IHC and FISH in the detection of HER2 amplification (P=0.019). Also, there was a significant difference between HER2 amplification and menopause in patients (P=0.035).
Conclusion: This result demonstrated that the IHC test is not a reliable test to determine HER2 amplification. This study represented that FISH analysis is more reliable than IHC and must be preferentially performed for all cases, especially for HER2 +2 cases for whom the IHC result is 2+.

Keywords: Breast cancer; Fluorescence in situ hybridization (FISH); Human epidermal growth factor receptor-2 (HER2); Immunohistochemistry

INTRODUCTION

Breast cancer is one of the most common cancers and the leading cause of death from lung cancer in women[1]. According to global statistics, more than 1,2 million new breast cancer patients are diagnosed annually and over half a million people die from the disease[1,2]. Breast cancer is increasing in developing

countries than in other countries[3]. In Iran, the prevalence of breast cancer is high among women, accounting for 21.4% of all cancers. It's also a decade earlier, demonstrating the importance of screenings for breast cancer in Iran[4]. In recent years, studies to determine predictive factors have improved dramatically. Clinical and pathological features such as tumor size, lymph node involvement, and histologic grade are important in determining the patient's clinical course. In addition, recent molecular markers of estrogen (ER) and progesterone (PR) receptors, tumor suppressor protein p53, and Ki67 cell proliferative marker have been widely used to determine the prognosis of breast cancer and have implications for therapeutic strategies[2]. One of the most important biomarkers that are considered in the prognosis and treatment of breast cancer is the HER 2 / neu or c-erb-B2 gene located on chromosome number 17[5]. If this gene carries a mutation, and amplification (Amplification) is converted to Her2 oncogene which causes uncontrolled growth and cell division, it normally encodes a protein receptor that appears on the cell surface and promotes cell proliferation by binding to growth factors and inducing tyrosine kinase activity. Overexpression of HER 2 is found in about one-third of breast cancer cases and accordingly, an anticancer drug called Herceptin, an antibody against the receptor Her2, is actually produced[1].Due to the high cost of Herceptin supply, proper selection of patients who will benefit from this drug is very important. On the other hand, improper use of Herceptin imposes a heavy burden on subsidized persons as well as on paying government agencies[6]. Additionally, Herceptin has hazardous side effects, causing heart dysfunction and severe heart complications such as congestive heart failure in 5-15 percent and 1-4 percent of people, respectively. Therefore, the proper use of this drug leads to appropriate treatment and improved prognosis in HER 2 positive patients. On the other hand, it reduces the cost of treatment to the family and the health system and also does not impose side effects on people who do not benefit from the drug[7].There are generally two ways to measure HER 2 levels: Qualitative immunohistochemical (IHC) assay and fluorescence in situ hybridization (FISH) method, which is a more accurate and quantitative method. FISH is a standard and original test, which examines the number of copies of the HER2 gene in the nucleus of cancer cells[6]. The IHC technique examines the expression status of HER 2 protein at the surface of cancer cells and is recommended as the first-line of studies in patients because of its high sensitivity and relatively low cost. However, different studies have reported varying levels of IHC accuracy in the diagnosis of breast cancer[1,7]. The purpose of this study was therefore to compare the methods of IHC and FISH in the accurate diagnosis of HER2 gene overexpression in patients with paraffin-embedded breast cancer.

Materials and Methods
Materials
Rabbit Anti-human c-erbB2 Monoclonal Antibody (Dako A0485, Denmark); Liquid DAB+Substrate Chromogen System (DakoK3468, Denmark); Target Retrieval Solution (DAKO S2367, Denmark); Hematoxylin (Panreac, Spain); Primary and secondary antibody (Master Diagnosis, Spain), Hydrogen peroxide, Methyl alcohol, Entelan glue, Ethyl alcohol 99.6%, NaCl, EDTA and Tris-(hydroxymethyl)aminomethane (All from Merk, Germany); Ethyl alcohol96-70% and Xylene (Shiminab, Iran); Pepsin (Sigma, Germany). All used experimental apparatus were standardized and calibrated.

Patient Samples
In this descriptive-analytical study, 44 paraffin blocks with breast carcinoma were collected from the specimens of patients admitted to the pathology laboratory of Imam Reza Hospital in Kermanshah. The slides provided from the samples' paraffin blocks were stained using the conventional hematoxylin and eosin method. The minimum sample size required to determine the sensitivity and specificity of IHC testing particularly in comparison to FISH was calculated using statstodo online software (http://www.stastodo.com/SSizSenSpc_Pgm.php) as follows:
The sample size for Sensitivity or Specificity
Probability of Type I Error (alpha) =0 .05
Power (1-bta) = 0.9

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Sen or Spc in Grp 1 = 0.78
Sen or Spc in Grp 2 =1
The sample size required (per group) for unpaired comparison =42
The sample size required for paired comparison (Minimum) =36
Sample size required for paired comparison (Maximum) =36

Hematoxylin and Eosin (H and E) Staining
Paraffin-embedded tissues were stained with hematoxylin and eosin (H and E) method as 4 μm sections. In this technique, glass slides containing sections of tissue incubated for 2 hours at 70 ° C. Slides were then soaked in several containers filled with xylene, the graded sequence of ethanol solutions, hematoxylin, lithium carbonate, and eosin.

Immunohistochemistry
Immunohistological staining has been performed on formalin-fixed paraffin sealed tissue sections using Her2 antibodies. For this reason, 4μm sections of tissue were deparaffinized in a hot air oven for 24 hours at 60-65 ° C. The slides were then rehydrated for approximately 45 minutes in xylene and a graded sequence of ethanol solutions. Slides were immersed in Tris buffer jar (pH = 9) and warmed for 20 minutes in the autoclave at 121 ° C followed by washing in PBS solution to retrieve antigens. In order to decrease intracellular peroxidase activity, slides were soaked in a solution of 3% Hydrogen peroxide in methanol for 15 minutes, washed with PBS for 10 minutes. Following washing with PBS, the slides were incubated with primary and secondary antibodies at 45-120 and 30-45 minutes, respectively in a humid and dark place at room temperature. The slides were washed in PBS and coated with a chromogenic surface solution known as as3,3'-diaminobenzidine (DAB) for 5 minutes. The counterstaining was performed for 30-60 seconds with hematoxylin and washed in water. Stained slides were immersed in graded ethanol series and then xylene for transparency and dehydration of tissues. Afterwards, slides were mounted under a microscope for examination. Negative controls were applied to antibody diluents to remove the primary antibody. Her2 positivity was assessed based on guidelines from the American Clinical Oncology Society / American Pathology College 2013 Her2 check criteria for breast cancer[8]. All specimens were first visualized by one pathologist and then by three pathologists simultaneously by a ten-eye microscope (Nikon, Japan), and, based on staining specimens, samples scored as 0/+ 1 were considered negative, scores as 3+ were considered positive, and those scored 2+ were regarded suspicious (Table 1). Samples were then sent to a reputable laboratory for FISH testing as a gold standard to be evaluated using a dedicated HER2 fluorescent probe.

Statistical analysis
Data were analyzed using SPSS (V.21). Chi-square analyses tested the correlation between Her2 expression and estrogen receptor and other variables. All differences at $P < 0.05$ were considered statistically significant. The IHC results were compared with FISH using the Contingency Coefficient Cramer's V and Cohen's kappa tests.

RESULTS
Forty-four patients were included in the study. The age, family history, and other characteristics of patients are shown in Table 2. In the FISH technique (Figure 1), HER2 + was detected in 21 (47.7%) out of 44 patients, and HER2- was detected in 23 (52.3%) patients. Similarly, in the IHC technique (Figure 2), 3 (6.8%) patients had IHC 3 + (positive), and 5 (11.4%) had IHC 0 and IHC +1 (Negative), and in 36 (81.8%) patients, IHC 2+ (Equivocal) was statistically significantly different from standard FISH method (P = 0.019) (Table 3).
As Table 4 shows, the HER2 gene is significantly more amplified in patients who entered menopause than in other women (P = 0. 035), and there was no statistically significant association between HER2 gene proliferation and estrogen, progesterone receptors, or the P53 and KI67 genes (Table 5).Table 5 shows the differences in IHC test results reported by one and three pathologists. As the Table shows, when the pathological slides were reviewed by three pathologists, the specificity was upgraded from 13.04% to 21.74%, and the positive predictive value decreased from 51.22% to 50%.

Table 1: HER 2/neu scoring in breast cancer

0	Negative no reactivity or no membranous reactivity in any tumor cell	Negative
1+	Tumor cell cluster with a faint/barely perceptible membranous reactivity irrespective of percentage of tumor cells stain	Negative
2+	Tumor cell cluster with a weak to moderate complete, basolateral or lateral membranous reactivity irrespective of percentage of tumor cells stained	Equivocal
3+	cell cluster with a strong complete, basolateral or lateral membranous reactivity irrespective of percentage of tumor cells stained	Positive

Table 2: Demographic characteristics of patients with breast cancer

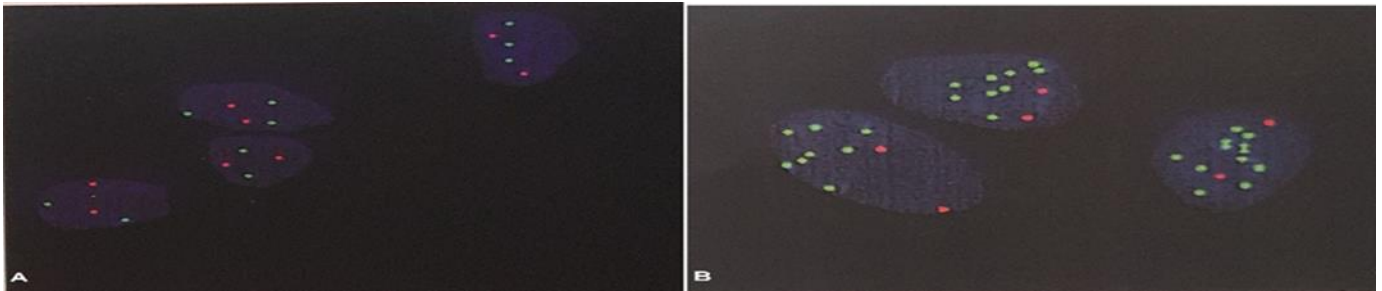|	Variable name		Frequency (percent)
1	Age	Less than 45 years	23 (52,3%)
	More than 45 years		21 (47,7%)
2	family history	Positive	11 (25%)
	Negative		21 (47,7%)
	Unknown		12 (27,3%)
3	Menopause	Positive	16 (36,3%)
	Negative		28 (63,7%)
4	Marital status	Married	7 (15,9%)
	Single		37(84,1%)

Figure 1. FISH images of a HER 2gene amplified sample using HER 2probes and centromere chromosome 17(red). A: Cores with only two green signals are normal. B: HER 2gene is amplified in the tumor cells.

Figure 2. IHC images of a HER2 gene amplified sample using anti-HER2 antibodies. A: It's normal. B: Suspected HER2 gene. C: HER2 gene is proliferated in tumor mature cells

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Table 3: Concordance between results of the HER2 gene amplification report by IHC and FISH methods

IHC (%)	FISH		
	Cramer's V 0.019	Negative number (%)	Cramer's V 0.019
Negative	5 (11.4%)	5 (21.7%)	5 (11.4%)
Suspect	36 (81.8%)	18 (78.3%)	36 (81.8%)
Positive	3 (6.8%)	0 (0.0%)	3 (6.8%)
Total Number	44 (100%)	23 (100%)	44 (100%)

Table 4: Comparison of menopausal status of patients with HER2 gene proliferation

Menopause		HER2 (FISH)			P
		Women in menopausal age	Women of non-menopausal age	Total	
	Positive	11(52.4%)	5 (21.7%)	16 (36.4%)	
	Negative	10 (47.6%)	18 (78.3%)	28 (63.6%)	0. 035
		21 (100%)	23 (100%)	44 (100%)	

Table 5: Effect of Estrogen and progesterone receptors, P53 Gene and Ki67 antigen on the tumor cell surface of patients

		HER2 (FISH)			P
Estrogen Receptor(ER)		Her2 neu+	Her2 neu-	Total	
	Positive/ER	16(76.2%)	20 (87%)	36 (81.8%)	0.355
	Negative/ER	5 (23.8%)	3 (13%)	8 (18.2%)	
		21 (100%)	23 (100%)	44 (100%)	
Progesterone Receptor(PR)		Her2 neu+	Her2neu-	Total	
	Positive/PR	15(71.4%)	18 (78.3%)	33 (75%)	0.601
	Negative/PR	6(28.6%)	5 (21.7%)	11 (25%)	
		21 (100%)	23 (100%)	44 (100%)	
P53 gene		Her2neu+	Her2neu-	Total	
	Positive/p53	14(66.7%)	14 (60.9%)	28 (63.6%)	0.690
	Negative/p53	7(33.3%)	9 (39.1%)	16 (36.4%)	
		21 (100%)	23 (100%)	44 (100%)	
Ki67 antigen		Her2neu-	Her2neu-	Total	
	Positive/Ki67	21(100%)	22 (95.7%)	43 (97.7%)	0.334
	Negative/Ki67	0(0%)	1 (4.3%)	1 (2.3%)	
		21 (100%)	23 (100%)	44 (100%)	

Table 6. Differences in IHC test characteristics reported by one and three pathologists

	Observed by a pathologist	Observed by three pathologists
Sensitivity	100%	100%
Specificity	13,4%	21.7%
Positive predictive value	51.22%	50%
Negative predictive value	100%	100%

DISCUSSION

Investigation of prognostic factors in Iranian women with breast cancer has recently received the attention of researchers[3]. However, the present study is the first study to compare IHC and FISH methods in the diagnosis of HER2 gene overexpression in patients with breast cancer in western Iran. In recent studies, HER2/neu gene proliferation was usually associated with higher grade and larger tumor size, lymph node involvement, distant metastasis, estrogen receptor deficiency, greater recurrence rate, and poorer prognosis[9]. The overexpression of the HER 2 gene may also be useful in predicting which patients will benefit most from chemotherapy or hormone therapy[3,10]. However, in this study, there was no significant correlation between HER2/neu gene expression and estrogen receptor, progesterone, KI67 and p53 genes. The results of this study are consistent with some similar studies. This include the results of Huang's study[11] in China in which overexpression of HER2 did not correlate with hormone receptors. Moreover, in another study in Tehran by sirati[11,12], HER2 was not significantly correlated with any of the ER, PR and p53 biomarkers. One of the reasons for these conflicting and sometimes contradictory findings is that laboratories use different specificity and sensitivity reagents, varying methods of detection, and different scoring. Furthermore, for positive or negative samples, many laboratories use optional definition systems[1]. Since the increased expression of HER2/neu receptor protein at the cell surface is due to gene proliferation, it is expected that the status of the HER2 gene and protein will be consistent with each other[6]. In this study, 21 patients (47.7%) were HER 2 + and 23 patients (52.3%) were HER2- by examining the HER2 gene by FISH technique. Whereas the IHC technique had 3 (6.8%) HER2 + patient and 5 (11.4%) HER2-, and 36 (81.8%) patients were suspected of HER2 status which was statistically significant (P = 0.019). Usually, the amount of HER2 protein present in the sample is measured in laboratories using the IHC method. When IHC testing is negative, no further action is needed.

Some HER2 genotypes such as chromosome 17 polymorphism and genomic heterogeneity may lead to inconsistencies in IHC and FISH results that are clinically relevant[13]. It is recommended that in cases where the staining intensity is not high such as +2 positive IHC results it should be repeated with FISH to determine the true status of HER 2[1]. Of course, both IHC and FISH techniques are complementary in nature, each of which provides a specific aspect of the pathology of breast cancer[14].The IHC technique determines the increase in cell surface receptor protein expression and while the FISH technique tests the HER2 gene status within the cell nucleus. Both techniques have limitations and can be used to detect better and more accurately[1].

IHC is easily done in laboratories, but the findings are not definitive, and FISH is a very sensitive and precise method for determining the amplification of HER 2 genes[7]. Nevertheless, besides being more costly than the IHC, the FISH method requires specialized tools and specialist resources that are not readily available in laboratories and require high costs. In addition, tumor morphology is not optimized in FISH and it is difficult to differentiate different carcinomas. The FISH method also has the drawback that fluorescent signals at room temperature quickly fade, making it difficult for potential comparison to being preserved[1].

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