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HER2/neu Status in Breast Cancer Specimens: Comparison of Immunohistochemistry (IHC) and Fluorescence *in situ* Hybridization (FISH) Methods

Estado del HER2/neu en Especímenes con Cáncer de Mama: Comparación entre los Métodos de Inmunohistoquímica (IHC) y Fluorescencia de Hibridación In Situ (FISH)

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SUMMARY: HER2 amplification or overexpression is considered as disease outcome and a predictive marker of response to treatment in breast cancer. The present study aimed to compare the results of IHC and FISH for determining HER2 and to search the interpretational differences. Samples (n= 169), of which 31 were the paraffin blocks sent from outer centers, that underwent FISH analysis for HER-2 were included. Samples were re-reviewed by IHC in our laboratory. FISH test was negative in 131 (77.5%) and positive in 38 (22.5%). When those with previous IHC 0-1+ were re-reviewed, the results were found again 0-1+ and none of them was FISH positive. Inconsistency between re-reviewed IHC and previous IHC results was 25% for those with 2+ score and 11% for those with 3+ score. Consistency between IHC and FISH was 17% and 67% for previous IHC 2+ and 3+, respectively, whereas it was 23% and %75 for re-reviewed IHC 2+ and 3+, respectively. Whilst 79% of the samples evaluated as 2+ by the inexperienced pathologist were found to be 0-1+ on the re-review, all of them were FISH negative. According to our results, we suggest that samples with IHC 2+ should be re-reviewed by consulting with an experienced pathologist.

KEY WORDS: Breast cancer; HER2; Immunocytochemistry; FISH technique.

INTRODUCTION

Human epidermal growth factor receptor protein-2 (HER2/neu; c-erb-2) gene is located on human chromosome 17 and encodes the HER2 protein. HER2 overexpression is observed in about 15%-20% of breast cancer patients (Jacot *et al*., 2013; Mukai, 2010; Ross, 20099). HER2 amplification or overexpression is considered as disease outcome and as the predictive marker of response to treatment (Jacot *et al*.; Bravatà *et al*., 2013). HER2-positive tumors are usually resistant to cytoxan- or tamoxifen-based regimens, but sensitive to anthracycline-based chemotherapy and trastuzumab therapy. The trastuzumab antibody binds to the extracellular domain of HER2, which is a transmembrane tyrosine kinase receptor, and then growth inhibition and apoptosis of tumor cells overexpressing HER2 occur (Bravatà *et al*.). Trastuzumab has made substantial contributions to the adjuvant treatment of HER2-positive breast cancers. Studies carried out with second generation drugs are ongoing (Jelovac & Wolff, 2012). With the advent of therapy to target the HER2/neu protein, it has become a requested marker to identify patients appropriate for chemotherapy with the anti-HER2 monoclonal antibody trastuzumab.

Various commercially available kits or in-house protocols are used to determine HER2 status and comparison studies have been conducted (Cayre *et al*., 2007; Manion *et al*., 2011; O'Grady *et al*., 2010). For the determination of HER2/neu protein expression, immunohistochemistry (IHC) is an exclusively used method and almost a standard part of pathology reports in invasive breast cancer. In cases of equivocal (2+) IHC results or without previous IHC, FISH techniques are used for detecting HER2 gene amplification. Fluorescence in situ hybridization (FISH) has been reported to be a method of choice to detect the candidates for HER2-targeted therapy in terms of accuracy, reproducibility, and predictivity (Sauter *et al*., 2009). Ellis *et al*. (2005) reported that the use of IHC and FISH methods in combination was the most effective strategy even though it was not cost

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effective. The aim of the present study was to compare the results of IHC and FISH in our laboratory and to search the interpretational differences.

MATERIAL AND METHOD

All patients with invasive breast cancer, who underwent FISH analysis for HER-2 between 2009 and 2012 in our department, were included in the study. Of 169 samples included in the study, 31 were the paraffin blocks sent from outer centers for FISH analysis.

Immunohistochemistry. Immunohistochemical staining for HER2/neu was performed on 4 μm sections of formalin-fixed, paraffin-embedded tissues. Sections were stained using Ventana HER2 kit (Pathway Anti-Her2/neu (4B5), Ventana Medical Systems Inc., Tucson, Arizona USA) following the manufacturer's guidelines. The same tissue blocks were used for both IHC and FISH.

Fluorescence in situ hybridization. Following the preparatory steps and pretreatment, denaturation and hybridization was performed on day 1. ZytoLight® SPEC HER2 /CEN17 dual color probe kit (Zytovision Molecular Diagnostics Simplified, Bremerhaven Germany) was used for hybridization. The slides were hybridized overnight at 37 ˚C. On day 2, following the washing steps, DAPI/ Antifade-solution (MT1) solution was dropped onto the slides. Evaluation of the slides was carried out by Olympus BX51 fluorescent microscope. With the use of appropriate filters, the signals of labeled HER2 gene (green) and alpha-satellite-sequences of the centromere of chromosome 17 (red) were counted on 100 cells for each sample.

Interpretation of the IHC slides that was previously evaluated by different pathologists in our department, were re-reviewed by two pathologists with 25 years of experience in breast pathology and blind to the first results. Consensus of these two pathologists was considered as a final decision. The results of experienced pathologists were assigned to Group A, the results of other pathologists were assigned to Group B, and the results of the tissues sent from outer centers were assigned to Group C. FISH evaluation and scoring was performed by the same experienced pathologists.

For the evaluation of HER-2 overexpression by IHC and FISH, the United States Food and Drug Administration (FDA) and the American Society of Clinical Oncology/ College of American Pathologists (ASCO/CAP) guidelines were used (Wolff *et al*., 2013).

RESULTS

In the present study, samples from 169 patients with invasive breast cancer, who underwent FISH analysis for HER2/neu, were re-evaluated. The mean score of samples that underwent FISH analysis was 1.93 (ranged between 0.4 and 15.0). The results were negative in 131 samples (77.5%) and positive in 38 samples (22.5%). HER2/neu IHC results were available for 138 samples. All results are summarized in Table I.

Table I. HER2/neu FISH and IHC results.

	HER2/neu	n (%)
FISH (n=169)	Negative	131 (77.5)
	Positive	38 (22.5)
IHC (n=138)	0: Negative	8 (5.8)
	1+: Negative	6 (4.2)
	2+: Equivocal	115 (83.3)
	3+: Positive	9 (6.5)

Of the samples with HER2/neu IHC results of 0-1+, 100% were FISH negative. Of the samples with IHC 2+ and IHC 3+, 17.4% and 66.7% were FISH positive, respectively (Table II).

Table II. Comparison of the results of HER2/neu IHC and FISH analyses (n= 138).

	FISH (-)	FISH (+)
IHC	**n (%)**	**n (%)**
0-1+	**14 (100.0)**	0 (0.0)
2+	95 (82.6)	**20 (17.4)**
3+	3 (33.3)	**6 (66.7)**

Comparison of the IHC results evaluated by experienced pathologist and inexperienced pathologist is demonstrated in Table III. Comparison of the IHC results evaluated by experienced pathologist and FISH results is presented in Table IV. Of the samples with IHC 0-1+, which was evaluated by experienced pathologist, 100% were FISH negative. However, of the samples with IHC 2+ and 3+, 23.3% and 75% were FISH positive, respectively.

Table III. IHC results evaluated by experienced pathologist and inexperienced pathologist (n= 138).

	Re-reviewed IHC		
Previous IHC	**0-1+**	**2+**	**3+**
	n (%)	**n (%)**	**n (%)**
0-1+	**14 (100.0)**	0 (0.0)	0 (0.0)
2+	29 (25.2)	**86 (74.8)**	0 (0.0)
3+	1 (11.1)	0 (0.0)	**8 (88.9**

Table IV. Re-reviewed HER2/neu IHC and FISH results (n= 138).

Re-reviewed IHC	FISH (-) n (%)	FISH (+) n (%)
0-1+	**44 (100.0)**	0 (0.0)
2+	66 (76.7)	**20 (23.3)**
3+	2 (25.0)	**6 (75.0)**

Comparison of previous IHC results with the final evaluation and interpretation differences of pathologists are presented in Table V; re-reviewed IHC results of the blocks from outer centers are also presented. When the samples that were 2+ in the previous HER2/neu IHC analysis were re-reviewed, 82.1% of those in Group A, 21.4% of those in Group B, and all of those in Group C were found to be 2+ again.

Table V. Comparison of previous and re-reviewed IHC results.

Pathologist	Previous IHC	Re-reviewed IHC 0-1+ n (%)	2+ n (%)	3+ n (%)
A	**0-1+**	**14 (100.0)**	0 (0.0)	0 (0.0)
	2+	14 (17.9)	**64 (82.1)**	0 (0.0)
	3+	0 (0.0)	0 (0.0)	**8 (100.0)**
B	**2+**	15 (78.9)	**4 (21.4)**	0 (0.0)
	3+	1 (100.0)	0 (0.0)	**0 (0.0)**
C	**2+**	0 (0.0)	**18 (100.0)**	0 (0.0)

A= Experienced pathologist; B= Inexperienced pathologist; C= Tissues from outer centers.

Comparison of IHC and FISH results among pathologist groups are presented in Table VI. Gene amplification could not be observed via FISH analysis in any of the samples that were evaluated as 2+ and 3+ by the pathologist in Group B. Of the samples evaluated as 2+ and 3+ by thepathologist in Group A, 15.4% and 75% were FISH-positive, respectively. The rate of FISH-positivity was 44.4% in 2+ samples in Group C.

Table VI. Comparison of IHC and FISH results among pathologist groups.

Pathologist	Previous IHC	FISH (-) n (%)	FISH (+) n (%)
A	**0-1+**	**14 (100.0)**	0 (0.0)
	2+	66 (84.6)	**12 (15.4)**
	3+	2 (25.0)	**6 (75.0)**
B	**2+**	19 (100.0)	**0 (0.0)**
	3+	1 (100.0)	**0 (0.0)**
C	**2+**	10 (55.6)	**8 (44.4)**

DISCUSSION

IHC method for testing HER-2/neu expression is a standard procedure in our laboratory and a part of our pathology reports in invasive breast carcinomas. FISH is the subsequent and gold method in equivocal (2+) IHC cases.

It is controversial which method is the best for HER2/neu determination. Available methods evaluate HER/neu through different aspects. Whilst IHC method evaluates protein overexpression on cell surface in tissue samples, FISH method determines the number of copies of the gene that codes this protein. Enzyme linked immunosorbent assay (ELISA) method measures serum antigen levels (Yeh, 2002). In the present study, IHC and FISH methods were performed using FDA-approved kits and the results were compared.

Being rapid and technically easier and cheaper are the main advantages of IHC; however, evaluation is subjective and shows interobserver variability. Moreover, pre-analytic and analytic processes influence immunoreactivity. FISH gives quantitative results with lower interobserver variability. Nevertheless, FISH is a more time-consuming method, is difficult to apply, and requires trained technician. It is more expensive since it requires test kits and a special microscope and stained preparations cannot be archived.

Whilst 0-1+ (HER2 negative) and 3+ (HER2 positive) IHC results show higher correlation with FISH, consistency of 2+ results (HER2 equivocal) with FISH is contradictory (Yeh). FISH analysis is recommended for all samples that are found 2+ and 3+ by IHC (Kovács & Stenman, 2010). Gene amplification could be demonstrated only in some of the samples that are found 2+ by IHC (Yeh). Studies have reported that FISH positivity in IHC 2+ samples varies between 7% and 89% (Ciampa *et al*., 2006; Rossi *et al*., 2006; Kuo *et al*., 2007; Sui *et al*., 2009; Goud *et al*., 2012; Zhang *et al*., 2012). Such diverse results might be due to the differences in the number of patients included in studies, centers' and/or pathologists' experience, and the test kits used.

Zhang *et al*. evaluated FISH results in 528 breast cancer samples with IHC 2+. Of these samples, 65.5% of IHC 2+ patients were negative for HER-2/neu amplification, 29.0% were positive, and the remaining was equivocal. They emphasized that FISH analysis was necessary in the samples with IHC 2+. Ciampa *et al*. demonstrated that of the samples

with IHC 3+, 52% were FISH positive and 48% were FISH negative. However, samples with IHC 2+ were more discordant with FISH, of which, 93% were FISH negative. FISH was found positive in two (9.5%) of 21 samples that were IHC 0-1+. Goud *et al*. investigated HER2/neu status by IHC and FISH in 90 breast cancer tissues. Thirty samples yielded negative results with both methods. Of 28 samples with IHC 2+, 20 were FISH positive and 3 were FISH negative, whereas 5 equivocal samples were found to be positive with repeated FISH testing. FISH was positive in 25 and negative in 7 of 32 samples with IHC 3+. They also suggested that all samples with IHC 2+ or higher should be analyzed also by FISH. Rossi *et al*. performed IHC and FISH in 81 samples and reported that IHC score and FISH evaluation showed a significant concordance (concordance coefficient 0.64). Gene amplification was not observed in any of the samples with IHC 0-1+. Besides, gene amplification was detected in 20% of cases with IHC 2+ and 77.78% of cases with IHC 3+. In the study performed by Sui *et al*., with 50 samples, the total concordance was found to be 82.0% (Kappa coefficient= 0.640, $p<0.001$). There was a high discordance in 30.0% of the patients with IHC 2+, 7.1% with IHC 3+, and 19.2% with IHC 0-1+. Kuo *et al*. performed a study with 54 samples and found that FISH was negative in all of IHC 1+ samples, whereas FISH was positive in 53% of IHC 2+ samples and 83% of IHC 3+ samples. Yaziji *et al*. (2004) evaluated 2,913 samples that underwent both IHC and FISH analyses and found positive predictive value of IHC 3+ score to be 91.6% and negative predictive value of IHC 0-1+ to be 97.2% when considered FISH as the standard test. The sensitivity of IHC was reported to be 92.6% when 2+ and 3+ scores were taken into account, whereas the specificity was reported to be 98.8% for IHC 3+ scores. Based on the results of the study, they recommended verification by FISH analysis only for IHC 2+ samples.

In brief, regarding the studies in the literature, consistency of IHC 0-1+ samples with FISH changes between 81% and 100% (Ciampa *et al*.; Goud *et al*.; Sui *et al*.; Kuo *et al*.). In the present study, all of the samples which were previously IHC 0-1+ were again found to be 0-1+ by reviewed IHC and FISH results were negative in 100% of these samples. In the literature, the FISH positivity in IHC 2+ samples is between 7% and 89% (Zhang *et al*.; Ciampa *et al*.; Goud *et al*.; Rossi *et al*.; Sui *et al*.; Kuo *et al*.). In the present study, the FISH positivity was 17% for previous IHC 2+ samples and 23% for re-reviewed IHC 2+ samples. Studies have been reported the consistency of IHC 3+ samples with FISH between 52% and 94% (Ciampa *et al*.; Goud *et al*.; Rossi *et al*.; Sui *et al*.; Kuo *et al*.). In the present study, consistency of IHC 3+ samples with FISH was 67% for previous IHC results, whereas it was found to be 75% for re-reviewed IHC results. In our department, re-reviewed IHC results showed higher consistency with FISH.

It is known that IHC can give different results due to subjective interpretation, samples were re-reviewed in our clinic. All of the previously IHC 0-1+ samples were again found 0-1+ when re-reviewed in our clinic. When previously IHC 3+ samples were re-reviewed in 88.9% was again 3+. Of the previous IHC 2+ samples, 25.2% were found 0-1+ when re-reviewed, whereas 74.8% were found 2+ again. The rate of consistency between previous and re-reviewed IHC results was determined to be 78.3%. When IHC results were re-reviewed according to the pathologists that performed previous evaluation, 86 (86%) of 100 samples evaluated by experienced pathologist were found to be consistent with the second evaluation. This consistency was 20% in the samples evaluated by inexperienced pathologist. When only 2+ samples were taken into account, the rate of consistency was 82.1% and 21.1% for experienced and inexperienced pathologists, respectively. Hoang *et al*. (2000), investigated interobserver reproducibility in IHC testing and assigned 100 samples to 4 different pathologists. Umemura *et al*. (2008), investigated inconsistency between IHC and FISH results among different laboratories (7 institutional and 3 commercial laboratories in Japan, one laboratory in Germany) and the reasons for this inconsistency. Concordance among the laboratories was good for IHC (k= 0.713) and excellent for FISH (k= 0.887). Discordance among the results was attributed to the evaluation process in 33.0% of the samples, staining procedures in 25.0%, and both evaluation and staining procedures in 41.7%.

In the present study, FISH was positive in 15.4% of the samples with IHC 2+ and in 75% of the samples with ICH 3+ evaluated by the experienced pathologist. Gene amplification was not observed in any of the samples evaluated as 2+ and 3+ by the inexperienced pathologist. In the study conducted by Perez *et al*. (2006), 2,535 samples were examined at local and central laboratories and samples with inconsistent result were additionally examined at the reference laboratory. Among the patients evaluated as strongly HER2 positive by local laboratories, a significant percentage of the patients were not confirmed by a central laboratory; 18.4% for IHC HercepTest and 11.9% for FISH. After re-evaluation of discordant samples in the reference laboratory, a high level of agreement between the central (Mayo Medical Laboratories) and reference laboratories was achieved as 94.3% for IHC (0, 1, and 2) and 95.2% for FISH.

In conclusion, FISH test was negative in 131 (77.5%) and positive in 38 (22.5%) of 169 samples. When those with previous IHC 0-1+ were re-reviewed, the results were found again 0-1+ and none of them was FISH positive. Inconsistency between re-reviewed IHC and previous IHC results was 25% for those with 2+ score and 11% for those

with 3+ score. Consistency between IHC and FISH was 17% and 67% for previous IHC 2+ and 3+, respectively, whereas it was 23% and 75% for re-reviewed IHC 2+ and 3+, respectively. Whilst 79% of the samples evaluated as 2+ by the inexperienced pathologist were found to be 0-1+ on the re-review, all of them were FISH negative.

Results of the present study supported the hypothesis that the consistency between IHC and FISH is higher for samples with 0-1+ and 3+ but variable for samples with 2+. Since there may be differences due to subjective interpretation with IHC analysis, we recommend that samples with 2+ should be re-reviewed by consulting with an experienced pathologist. Unnecessary requests for molecular test would be reduced by experienced pathologist by minimizing interpretation differences and by trained technicians and qualified techniques by minimizing evaluation errors.

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RESUMEN: La amplificación o sobreexpresión de HER2 es un marcador predictivo de la respuesta al tratamiento en el cáncer de mama y es considerada como resultado de esta patología. El presente estudio tuvo como objetivo comparar los resultados de IHC y FISH para la determinación de HER2 y buscar diferencias de interpretación. Las muestras (n= 169), de las cuales 31 eran bloques de parafina, fueron enviadas desde centros externos y sometidas a análisis FISH para HER-2. Las muestras fueron revisadas en nuestro laboratorio con la prueba IHC. La prueba FISH resultó negativa en 131 casos (77,5%) y positiva en 38 (22,5%). Cuando se re-examinaron aquellos casos con resultados previos de IHC 0-1+, se encontró que los resultados fueron iguales (0-1+) y ninguno de ellos fue positivo para FISH. Se encontró inconsistencia entre los casos previos y las nuevas revisiones con IHC y fueron del 25% para aquellos casos con puntuación 2+ y del 11% para aquellos con 3+ de puntuación. La consistencia entre IHC y FISH fue del 17% y del 67% para casos previos analizados con IHC 2+ y 3+, respectivamente, mientras que fue de 23% y 75% para los reanalizados con IHC 2+ y 3+, respectivamente. Mientras que en el 79% de las muestras evaluadas con puntuación 2+ por patólogo inexperto resultaron ser 0-1 + con la nueva revisión, todos estos casos fueron FISH negativos. De acuerdo con nuestros resultados, sugerimos que las muestras con puntuación 2+ de IHC deben ser re-evaluadas por un patólogo experimentado.

PALABRAS CLAVE: Cáncer de mama; HER2; Inmunocitoquímica; Técnica de FISH.

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