

Detection of HER-2/neu Amplification on Fine Needle Aspirates of Breast Cancer Using Fluorescence *in Situ* Hybridization*

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ABSTRACT

The accuracy of diagnostic assays for HER-2/neu in breast cancer is extremely important as HER-2/neu status is essential in tailoring adjuvant and/or neoadjuvant treatment in every patient. FNAC is widely practiced in Egypt in preoperative diagnosis of breast cancer for its low cost and high diagnostic accuracy. Since the determination of HER-2/neu protein expression on cytological preparations was previously found to be unreliable for clinical use, we opted for the assessment of HER-2/neu status in fine needle aspirates using FISH. The main objective of this study was to evaluate the reliability of HER-2/neu status assessment by FISH on fine needle aspirates of breast cancers by comparing the results with IHC and FISH on FFPE tissue sections obtained from corresponding surgically resected specimens. Fine needle aspirates from 40 breast cancer patients with pathologically confirmed breast cancer were included in the study. They were submitted for HER-2/neu evaluation by FISH. After surgery, the corresponding FFPE sections were evaluated for HER-2/neu by FISH and by IHC. FNAs from 11 cases proved to be amplified by FISH, while 29 cases were not amplified. Apart from two cases that showed lack of signals, all specimens evaluated by FISH on the corresponding FFPE sections showed matched results. The Measurement of Agreement between FISH on FNAs and FISH on FFPE sections was 86.7%, while that between FISH on FNAs and IHC was 72.5%. The high concordance rate in the present study between FISH evaluation of HER-2/neu gene amplification on FNAC samples and their corresponding FFPE samples indicate that FISH may be a reliable technique for HER-2/neu assessment on FNAs. Furthermore, FISH on FNAs gave us better hybridization signals than their corresponding FFPE tissue sections. Finally, we also conclude that all score (2+) cases by IHC should be reevaluated by FISH which is crucial for the patient management.

Keywords: Breast Cancer; FISH on FNA; Immunohistochemistry; HER-2/neu

1. Introduction

Breast cancer is one of the major health problems in Egypt and is by far the most common type of cancer in Egyptian women [1,2]. The development of breast cancer is associated with a number of genetic alterations involving the inactivation of tumour suppressor genes and the activation of oncogenes. A dominant mechanism, leading to oncogene activation is the amplification of specific genomic region [3].

The ERBB2 (HER-2/neu) oncogene is amplified and overexpressed in about 25% of invasive breast carcino-

mas. In general, ERBB2 amplification confers unfavorable prognosis, although its significance is less than that of the traditional prognostic factors—stage and grade [4]. There are two main methods for establishing HER-2/neu status in the diagnostic setting. HER-2/neu protein expression can be detected using immunohistochemistry (IHC), and HER-2/neu gene amplification can be quantified using in-situ hybridization (ISH) with either fluorescence (FISH) or chromogenic (CISH) probe detection [5]. The relationship between HER-2/neu protein levels and response to treatment is proportional to the degree of over expression [6,7]. Protein expression level correlates with HER-2/neu gene copy number, and IHC has been shown to be reliable in detecting increased protein expression in

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cases with a high level of gene amplification [8,9]. However scoring of HER-2/neu IHC is highly subjective and inter-observer reproducibility can be problematic, especially for 2+ cases [10].

FISH is a molecular technique performed on formalin-fixed paraffin-embedded (FFPE) tissues, frozen sections as well as fine needle aspirate biopsy (FNAB), which permits the direct visual assessment of gene copy number in interphase and metaphase nuclei [11]. DNA is more stable than protein, making FISH less fixation sensitive than IHC [12]. Gene amplification correlates directly with protein and mRNA levels, and FISH has been shown to be superior to IHC in predicting clinical outcome and response to trastuzumab therapy [13]. However, FISH has some disadvantages, one of them that it is much more expensive than IHC, although given the high cost of trastuzumab therapy, cost-effectiveness analysis has suggested that FISH testing all 2+/3+ cases and avoiding unnecessary treatment of false-positive cases may save money [14].

Preoperative determination of the HER-2/neu status is becoming increasingly important to plan for the type of surgery as well as for neoadjuvant chemotherapy in selected patients. Preoperative HER-2/neu testing can be done by IHC on FFPE core needle biopsies (CNB). Although the test was found to be successful on CNB [15], still some obstacles exist: The ASCO/CAP guidelines recommend that cores entirely involved by retraction artifacts or crush artifacts should not be used as a sample to perform/interpret HER-2/neu immunohistochemistry [16] and the UK recommendations precise that observers should be aware of the range of common artifacts, including edge artifacts, which can be problematic in small biopsy samples [17].

In Egypt, breast FNAB plays a major role in the diagnosis of both palpable and non-palpable breast masses because cost-efficient health care is a priority in this country and the less expensive but effective diagnostic methods are preferred. Furthermore Breast FNAB has proved to have a very goodspecificity, sensitivity, and diagnostic accuracy [18]. And therefore in our situation it would be useful to be able to identify HER-2/neu status by using FNAB specimens.

Unfortunately, HER-2/neu protein expression on cytological preparations with any of the fixatives was found to be insufficiently reliable for clinical use [18]. On the other hand, several studies have reported successful use of FISH on FNA cytological material to evaluate HER-2/neu amplification [19-23]. Yet, few studies compared the efficacy of FISH on FNA specimens and on corresponding FFPE tissue samples [18].

2. Objectives

The objective of this study was to evaluate the reliability

of HER-2/neu status assessment by FISH on FNAs of breast cancers by comparing the results with FISH and IHC done on FFPE tissue sections obtained from corresponding surgically resected specimens. We also aimed at evaluating concordance rates between IHC HER-2/neu positive and IHC HER-2/neu negative cases with FISH amplified and non-amplified cases respectively, in order to validate the HercepTest™ that is newly implemented in our laboratory.

3. Material and Methods

Between November, 2011 and May, 2012, fine needle aspirates were collected from 43 female patients having an American College of Radiology Breast Imaging Reporting and Data System (BIRADS) score of 5 on mammogram and undergoing preoperative confirmation of malignancy. All patients signed informed consents. Two FNA samples were subsequently discarded from the study because they were hypocellular, and one sample was also excluded because the post-operative biopsy showed an *in situ* ductal carcinoma.

Fine needle aspirates from the remaining 40 breast cancer patients were included in the study. They were submitted for routine diagnostic cytology as well as for HER-2/neu evaluation by FISH. After surgery, the corresponding sections of archival formalin-fixed, paraffin-embedded tissue were evaluated for HER-2/neu by FISH and by IHC.

3.1. Sample Preparation

1) FNA sample collection: Preoperative samples were collected by FNA under ultrasound guidance using a 23-gauge needle. Ultrasound guidance was used in all cases to ascertain the aspiration of sufficient amount of cells from within the tumor itself and not from any surrounding breast tissue. The aspirated material was smeared on glass slides, two of which positively charged. The 2 smears on positively charged slides were fixed in methanol/glacial acetic acid (3:1) and stored in -20°C until their use for FISH. The remaining slides were fixed in alcohol for routine H&E staining.

2) FFPE tissue sections: Postoperative tissue biopsies were fixed in 10% buffered formalin overnight and then were processed for paraffin embedding. Tissue blocks were then cut into 4 μm thick sections on positively charged slides, and incubated overnight at 56°C .

3.2. HER2/neu Immunohistochemistry

Immunohistochemical analysis was performed manually on FFPE tissue sections using the FDA-approved HercepTest™, according to the manufacturer's instructions.

Briefly, following deparaffinization and rehydration,

the tissue sections were subjected to heat-activated epitope retrieval in citrate buffer at 96°C for 30 minutes. Blocking of endogenous peroxidase preceded the addition of the primary anti-HER-2/neu antibody (rabbit anti-human c-erbB-2 A0485, Dako, Copenhagen, Denmark) at 1/100 dilution. Binding of the primary antibody was revealed by means of the chromogen substrate, 3,3'-diaminobenzidine (DAB). Slides were then counterstained with hematoxylin. A positive tissue control section was included in each run of immunohistochemical staining, as well as a negative control slide in which the primary antibody was omitted.

3.3. Scoring of Immunohistochemical Results

The slides were scored independently by 2 histopathologists (the first and the third authors) according to the ASCO & CAP guidelines [15] as follows.

3.3.1. Score 3 (Positive)

Uniform intense membrane staining of more than 30% of invasive tumor cells. A homogeneous (chicken wire) pattern should be present.

3.3.2. Score 2 (Equivocal)

Complete membrane staining that is non-uniform or weak but with obvious circumferential distribution in at least 10% of cells, or intense complete membrane staining in <30% of tumor cells.

3.3.3. Score 1 (Negative)

Weak, incomplete membrane staining in any proportion of invasive tumor cells, or weak, complete membrane staining in less than 10% of cells.

3.3.4. Score 0 (Negative)

No staining is observed in invasive tumor cells.

Before we started testing the patients included in the study we retrospectively tested 30 patients with known HER2/neu FISH status using the FDA-approved DakoHerceptTest™ for immunohistochemistry. Some technical issues were slightly modified as well as the interpretation criteria in order to meet the ASCO/CAP guidelines recommendations. A concordance rate of 90% was reached. The 10% of cases that showed lack of agreement (3 cases) were in the category of 2+ by IHC. All assay procedures were standardized before starting the new cases.

3.4. FISH Protocol

1) For cytological specimens: Slides were brought to room temperature and incubated in 2xSSC at 37°C for 30 min, gradually dehydrated in alcohol, air dried and proc-

essed by FISH.

2) For FFPE tissue sections: deparaffinization, pretreatment, enzyme digestion and fixation of slides were performed using the Vysis Paraffin Pretreatment Kit (Vysis) according to the manufacturer's recommended protocol.

3.5. Probe Application and Hybridization

Denaturation and hybridization were carried out in a HYBrite Denaturation/Hybridization System for FISH (Vysis). All slides were denatured at 72°C for 2 min. HER-2/neu probe mix (10 µl for FFPE sections and 3 µl for FNAC slides) was added and hybridization took place at 37°C for 14 - 18 h. The slides were then washed in post-hybridization wash buffer at 72°C for 2 min and counterstained with DAPI/antifade.

3.6. Evaluation of the FISH Signals

In each case, 100 - 200 well preserved, separately located interphase cells with clearly visible distinct signals were scored for HER-2/neu and chromosome 17 signals. FISH signals were analyzed using BX51/61 Olympus fluorescent microscope equipped with a suitable set of filters including: DAPI single band pass, dual band pass FITC/TRIC and triple band pass (FITC/TR/DAPI) (Olympus, UK LTD).

Only single, non-overlapping and intact nuclei were examined. Nuclei lacking hybridization signals were excluded from the evaluation. Results were expressed as a ratio of the numbers of copies of the HER-2/neu gene to the number of the chromosome 17 centromeric markers, with a ratio greater than two being considered amplified. Samples were classified as unamplified when two copies of HER-2/neu and two copies of chromosome 17 were found in the majority of cells and also when the HER-2/neu to chromosome 17 ratio was lower than two.

3.6.1. Positive

FISH HER-2/neu/CEP17 ratio 2.2 or greater, or FISH HER-2/neu gene copy number > 6.0.

3.6.2. Equivocal

FISH HER-2/neu/CEP17 ratio 1.8 - 2.2, or FISH HER-2/neu gene copy number 4.0 - 6.0.

3.6.3. Negative

FISH HER-2/neu/CEP17 < 1.8, or FISH HER-2/neu gene copy number < 4.0. (In conjunction with ASCO & CAP guidelines).

4. Results

HER-2/neu was evaluated by FISH on forty primary breast cancer FNAs and both by FISH and IHC on the corresponding paraffin sections.

The mean patient age was 42.35 years (range, 22 - 65 years).

All the included cases were diagnosed as infiltrating ductal carcinoma, NOS. Tumor size, grading and staging as well as lymph node status are shown in **Table 1**.

4.1. Patients' Criteria

The mean patient age was 42.35 years (range, 22 - 65 years). The largest group (21/40 (52.5%)) had Stage II disease, followed by Stage III (10/40, 25%), then Stage I (6/40, 15%)

Three (7.5%) of the patients had known distant metastases at the time of the surgery, and 27 cases (67.5%) were negative for lymph node metastases.

4.2. HER-2/neu FISH on FNAs

Out of the 40 FNAs, 11 (27.5%) were amplified, while 29 (72.5%) were unamplified.

In unamplified cases, two red signals and two green signals were frequently identified (**Figure 1**).

Amplified tumors, on the other hand, showed increased number of the red signals either singly or in clusters (**Figure 2**).

As regards the feasibility of FISH on cytological smears, hybridization was successful in all cases.

Table 1. Clinico-pathologic characteristics of the forty breast cancer cases.

Clinico-pathologic parameter	Number of the cases	Percentage
Tumor size		
<2 cm	6	15
2 - 5 cm	16	40
>5 cm	18	45
Tumor grading		
Well differentiated	4	10
Moderately differentiated	30	75
Poorly differentiated	6	15
Lymph nodes status		
Negative	27	67.5
Positive	13	32.5
Distant metastasis		
Absent	3	7.5
Present	37	92.7
Tumor staging		
I	6	15
II	21	52.5
III	10	25
IV	3	7.5

4.3. HER-2/neu FISH on FFPE Sections

Out of the 40 tumors examined, 9 cases (22.5%) were amplified (**Figure 3**), whereas 29 cases (72.5%) showed no signals amplification (**Figure 4**). Two cases were non-evaluable due to lack of signals. Amplification signals were more often distributed in clusters.

4.4. HER-2/neu Immunohistochemistry

On IHC, out of the same previous 40 tumors 24 (60%) were negative for the protein expression, while 16 cases (40%) showed overexpression: ten cases (62.5%) were scored as (3+) (**Figure 5**) and six cases (37.5%) as (2+) (**Figure 6**).

4.5. FISH on FNAs versus FISH on FFPE Sections

Apart from the two cases that showed lack of signals on

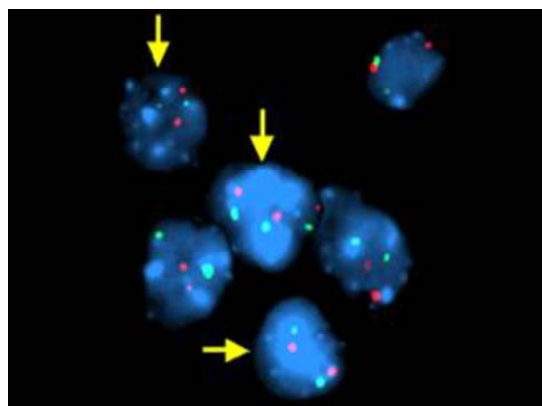


Figure 1. FISH image of a non-amplified Her 2-neu FNA breast cancer case showing normal pattern of 2 green and 2 red signals after hybridization with HER 2-neu probe. (Her2-neu red signals, centromeric green signals).

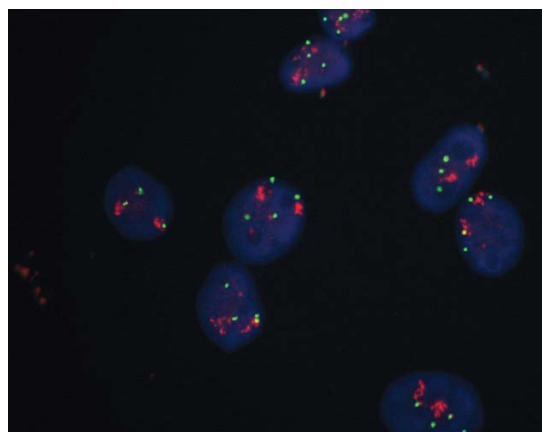


Figure 2. FISH image of an amplified Her 2-neu FNA breast cancer case showing increased number of red signals {HER-2/neu} than green signals {Cen 17}.

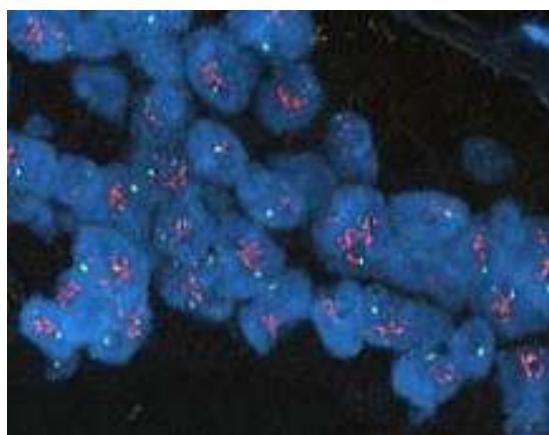


Figure 3. FISH image of the corresponding FFPE section of the FNA shown in Figure 2 with amplified HER-2/neu signals representing cluster pattern. Each tumour cell nucleus demonstrates HER-2/neu signals/CEP 17 signals ratio of >2.2.

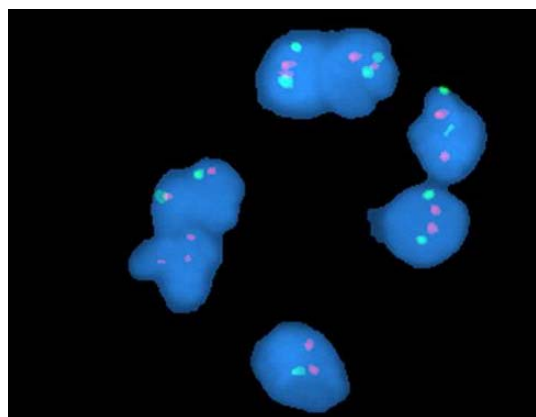


Figure 4. FISH image of the corresponding FFPE section of the FNA shown in Figure 1 showing non-amplified HER-2/neu signals. Normal pattern is obtained in most of the scored cells (2 green {cen 17} and 2 red {HER-2/neu} gene signals).

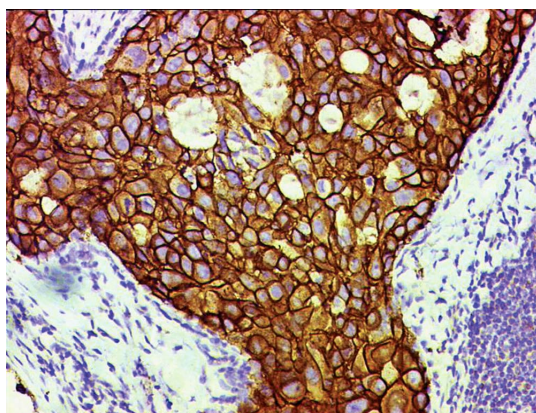


Figure 5. Positive immunostaining for HER-2/neu (score 3+). Uniform intense membrane staining in most invasive tumor cells is seen (Immunoperoxidase, $\times 400$).

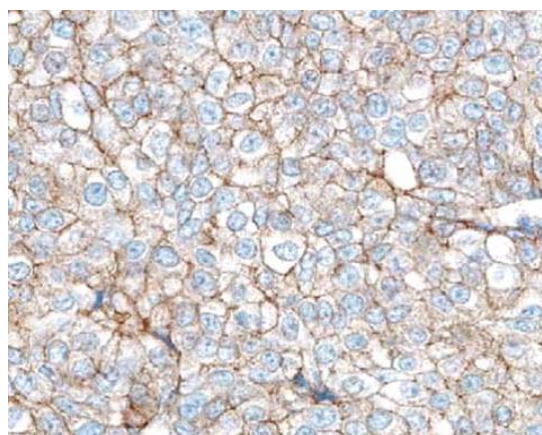


Figure 6. FFPE section showing score 2+ immunostaining for HER-2/neu, complete but weak membrane staining in more than 50% of tumor cells (Immunoperoxidase, $\times 400$).

FISH histology, all tumors evaluated by FISH on FNAs and on corresponding paraffin-embedded sections showed matched results.

The Measurement of Agreement (Concordance rate) between FISH on FNAs and FISH on FFPE sections was 86.7% as shown in **Table 2**.

4.6. FISH on FNAs versus IHC

All negative cases (score 0 and 1+) by IHC showed lack of amplification by FISH on FNAs. All (3+) cases by IHC were amplified by FISH on FNAs. As for the 6 cases scored as (2+) by IHC, only one of them showed amplification by FISH on FNA and the other 5 cases were unamplified. The Measurement of Agreement (Concordance rate) between FISH on FNAs and IHC was 72.5% as shown in **Table 2**.

4.7. FISH on FFPE Sections versus IHC

All negative cases (score 0 and 1+) by IHC showed lack of amplification by FISH on FFPE sections. Eight of the (3+) cases by IHC were amplified by FISH on FFPE sec

Table 2. HER-2/neu results comparing FISH on cytological samples with FISH and IHC on the corresponding histological sections.

FISH on FNA	FFPE sections			
	FISH		IHC	
	Amplified	Unamplified	Overexpressed	Not expressed
Amplified	9	2	11	0
Unamplified	0	29	5	24
Measurement of agreement	86.7%		72.5%	

tions. However, 2 of the (3+) cases by IHC were non-evaluable by FISH on FFPE sections. As for the 6 cases scored as (2+) by IHC, only one of them showed amplification by FISH on FFPE sections and the other 5 cases were unamplified. The Measurement of Agreement (Concordance rate) between FISH on FFPE sections and IHC was 89%.

5. Discussion

The accuracy of diagnostic assays for HER-2/neu in breast cancer is extremely important as HER-2/neu status is not only a prognostic marker but also predictive of response to chemotherapy, particularly to HER-2/neu-targeted therapy such as trastuzumab [24]. HER-2/neu data is also used to predict who will most likely benefit from doxorubicin, taxanes, tamoxifen and other reagents [25]. The diagnostic tests most widely used are IHC and FISH, measuring protein over expression and gene amplification, respectively [24].

Furthermore preoperative determination of the HER-2/neu status is becoming increasingly important to plan for the type of surgery as well as for neo adjuvant chemotherapy in selected patients.

FNAC is widely practiced in Egypt for the preoperative diagnosis of breast cancer and the preoperative detection of lymph node metastasis. Clinicians in our country continue to rely widely on FNAC and consider it an invaluable diagnostic tool. Core needle biopsy is done only in certain conditions [17]. Unfortunately, however the determination of HER2/neu protein expression on cytological preparations was found to be unreliable for clinical use [18]. We therefore opted for the assessment of HER2/neu status in fine needle aspirates using FISH, which was not tried before in our laboratory. By reviewing the literature, the feasibility of HER2/neu amplification detection by FISH in breast cancer FNAs was evaluated by few studies [18-23].

Our study proves the feasibility of HER2/neu amplification detection by FISH on FNA smears as hybridization was successful in all cases. From the technical point of view, FISH signals in cytological smears were more easily visualized than those on FFPE sections. The presence of isolated cells in smears made the signals identification and scoring easier than in tissue sections. This fact was previously reported by Klijanienko *et al.* [23] and by Bozetti *et al.* [19] who recommended the use of image analysis in evaluating histological samples. Furthermore, in our work FNA samples preparation for FISH didn't require pretreatment or enzyme digestion with the advantage of less expenses and shorter protocol time than their corresponding FFPE sections. As regards agreement of FISH on FNA smears of breast cancer cases with FISH on corresponding FFPE sections, apart from two cases

that showed lack of signals on FISH histology, all tumors evaluated by FISH on FNAs and on corresponding paraffin-embedded sections showed matched results. A good concordance rate of 86.7% was obtained in our study. If the 2 non-evaluable cases in FFPE sections were omitted from the calculations, then the concordance would be 100%. This is in agreement with Bozetti *et al.* [19] who found a concordance rate of 91% between FISH cytology and FISH histology and with Klijanienko *et al.* [23] who obtained a strong correlation ($P < 0.001$) between FISH on cytological and histological materials.

Furthermore, in our study, all negative cases (score 0 and 1+) by IHC showed lack of amplification by FISH on FNAs. All 3+ cases by IHC were amplified by FISH on FNAs. Similar results were obtained by McManus DT *et al.* [21] who detected high amplification by FISH on FNAs from 3 out of the 15 tumors they examined with matched diffuse membranous staining by IHC of the same tumors.

However, a concordance rate of only 72.5% was found between FISH on FNA smears and IHC in our study. This is attributed to the 6 cases that were given a score of (2+) by IHC and which gave variable results by FISH, as only one of them showed amplification by FISH on FNA and the other 5 cases were unamplified. In the present study, a 100% agreement was found between the (3+) cases by IHC and their corresponding FISH on FFPE sections except for 2 cases showing complete lack of signals. Similarly a 100% agreement was found between the score (0) and (1+) cases by IHC and their corresponding FISH on FFPE sections. However, the overall concordance rate between FISH on FFPE sections and IHC was 89%, owing to the variable agreement of the score (2+) cases with their FISH results.

This variable agreement of 2+ cases with gene amplification was previously encountered in several studies [18,26-28] emphasizing the importance of reevaluation of the HER-2/neu status by FISH in all (2+) cases.

Although several studies evaluating FISH and IHC support the view that FISH has a higher accuracy than IHC [29-31] and that FISH might be a better predictor of response to trastuzumab therapy [32,33], we still use IHC in our laboratory as the first line to triage cases and then the score (2+) ones are further evaluated by FISH. This is because of the lower cost of IHC which is covered by the medical insurance of the patient. Furthermore, IHC is easier to perform and the FISH equipment is not available in our routine pathology laboratory. Therefore we tried to validate the FDA-approved HercepTest that is newly implemented in our laboratory. After modification of some technical issues and interpretation criteria we compared the HercepTest results with the FISH results of the same cases. Only 90% concordance was reached before start-

ing this research and 89% with the research cases. The ASCO/CAP guidelines, however, recommend a minimum of 95% concordance for validation, which we couldn't reach due to the variable agreement of score (2+) cases by IHC. In fact HercepTest has been demonstrated in several studies to produce significant numbers of false positives [31,34-36].

In conclusion, the high concordance rate in the present study between FISH evaluation of HER2/neu gene amplification on FNAC samples and their corresponding FFPE samples indicate that FISH may be a reliable technique for HER2/neu assessment on FNA samples. This is of great significance in our institution where preoperative diagnostic FNAC is widely practiced. Evaluation of HER2/neu gene amplification on FNAC specimen may play a role in planning neoadjuvant chemotherapy in candidate patients, in the management of patients with advanced or recurrent disease and in deciding the type of surgery whether conservative or radical in selected patients. Furthermore, FISH on FNAs gave us better hybridization signals than their corresponding FFPE tissue sections. Finally, we also conclude that all score (2+) cases by IHC should be reevaluated by FISH which is crucial for the patient management. And if our hospital could afford it, we would recommend the use of FISH as the primary method for assessment of all FNAs and FFPE sections of breast cancers to determine the HER-2/neu status.

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