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Assessment of Hormone Receptor and Human Epidermal Growth Factor Receptor 2 Status in Breast Carcinoma Using Thin-Prep Cytology Fine Needle Aspiration Cytology FISH Experience From China

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Abstract: Estrogen receptor (ER) and progesterone receptor (PR) overexpression can be used to predict patient prognosis in breast cancer (BC). Human epidermal growth factor receptor 2 (HER2) is a reliable predictive marker in invasive breast cancer (IBC). Thin-Prep (TP) specimens are commonly utilized for immunocytochemistry (ICC) in fine needle aspiration cytology (FNAC). Thus, we sought to investigate if the incorporation of molecular diagnosis performed on TP-processed specimens is applicable in clinical practice.

Hormone receptors (HRs) and HER2 immunocytochemistry was performed on 542 primary breast cancer FNAC specimens using the TP method. One hundred fourteen *HER2* fluorescence in situ hybridization (FISH) analyses were performed on HER2 ICC 2+ FNAC specimens and the corresponding tissue samples. HRs results of TP slides and those of formalin-fixed paraffin-embedded (FFPE) slides were correlated well for ER (concordance rate = 93.3%, kappa value = 0.85) and PR (concordance rate = 88.6%, kappa value = 0.75). HER2 results for the TP slides and those of the matched FFPE slides also correlated well (concordance rate = 80.0%, kappa value = 0.62). The specificity of HER2 was 97.3%; however, the sensitivity was only 67.1%. Cytological specimens and histological samples showed a strong correlation (concordance rate = 99.1%, kappa value = 0.98) while being used to evaluate *HER2* gene amplification.

FNAC is a minimally invasive technique that can be used as an alternative method to collect tissue especially in cases where an excisional or core biopsy is difficult to obtain, or when recurrence is present. The results of ICC HRs in FNAC TP specimens may be used instead, but HER2 assessment may not be reliable enough for clinical use. FISH testing is necessary in this setting.

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Abbreviations: ASCO = American Society of Clinical Oncology, CAP = College of American Pathologists, CB = cell block, DCIS = ductal carcinoma in situ, ER = estrogen receptor, FFPE = formalin-fixed paraffin-embedded, FISH = fluorescence in situ hybridization, FNAC = fine needle aspiration cytology, HER2 = human epidermal growth factor receptor 2, HR = hormone receptor, IBC = invasive breast cancer, ICC = immunocytochemistry, IHC = immunohistochemistry, PR = progesterone receptor, TP = thin-prep.

INTRODUCTION

As a simple and rapid procedure in the diagnosis of breast cancers, fine needle aspiration cytology (FNAC) can provide a reliable and accurate approach for diagnosis of breast carcinoma. Meanwhile, it can be used as an alternative method to collect tissue from patients with inoperable, metastatic, or recurrent breast carcinomas. Immunocytochemistry (ICC) and molecular tests can also be performed on cytological materials to assess prognosis and predict treatment outcome of patients.

The Thin-Prep (TP) cytology (Hologic Corp., Marlborough, MA) procedure was recently developed for ICC and molecular diagnosis. Compared to conventional smears, a single specimen can generate multiple slides by using TP, and a minimal amount of reagent is required in the ICC procedure because the specimens tend to be small in size.[1–3]

Estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) are among those most well-established and extensively studied biomarkers for invasive breast cancer (IBC).[4–7] According to the guidelines published by American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP), immunohistochemistry (IHC) has been a commonly used method to detect the overexpression of hormone receptors (HRs) and HER2 in formalin-fixed paraffin-embedded (FFPE) tissue slides, while fluorescence in situ hybridization (FISH) is an alternative standard test for gene amplification of *HER2*.[8–10] Assessment of these biomarkers with ICC on FNAC specimens has been studied extensively on direct smears and cell block (CB) slides, with variably concordant rates.[11,12] However, very limited number of studies were conducted to evaluate the correlation of HR levels, HER2 protein overexpression, and gene amplification in FFPE tissues and TP slides of IBC.

The objective of this article was to detect if tissue collected with FNAC and processed using the TP method can be an alternative specimen for molecular diagnosis (HRs and HER2 status assessment) in primary and metastatic IBC.

MATERIALS AND METHODS

Patients

A total of 542 patients were enrolled into this study. This population included patients who were diagnosed with breast carcinoma from June 2010 to July 2013 at the Cancer Hospital of the Chinese Academy of Medical Sciences, Beijing, China. All patients were subjected to diagnostic FNAC by standard technique, with subsequent cytological evaluation.

FNAC biopsies were performed with 22 gauge needles. Four to 5 TP slides were prepared on the basis of the number of cells of different samples. One of them was used for HER2 detection and 2 of them were used for HRs, both by ICC method. The remaining specimens were stored at -80°C for additional *HER2* gene test, if necessary (see below).

All 542 TP slides have the corresponding histologic specimens for comparison and analysis. The histological samples were fixed in 10% neutral-buffered formalin for 6 to 72 hours.[9] Staining and diagnosis were performed in terms of the routine procedures. Three tissue sections were utilized for IHC evaluation of HRs and HER2. One tissue section was used for FISH, if it was deemed necessary.

This study is retrospective and the data were analyzed anonymously. No images and private information of the patients were released. The Institute Review Board of the Chinese Academy of Medical Sciences, Beijing, China approved the study protocol and agreed to waive the need for consent by the patient.

ER, PR, and HER2 Immunocytochemistry

Immunocytochemical staining for HRs was performed on FNAC TP preparations and on tissue sections with the avidin–biotin–peroxidase method, using the anti-ER, anti-PR, and anti-HER2 antibodies (mouse monoclonal antibody, procured by Zymed, San Diego, California, USA).

TP preparations were firstly fixed in 95% alcohol for 30 minutes. After the slides were taken out and dried, they were incubated with 3% H_2O_2 and then with normal serum overnight at 4°C with the primary antibody. The slides were then incubated with the biotinylated secondary antibody (Zymed, Invitrogen Corp., CA), and conjugated the avidin–biotin–peroxidase complex (PV9000, Zymed). The reaction was developed using 3,3′-diaminobenzidine (Zymed). With regard to the tissue slides, they were firstly deparaffinized in xylene and then rehydrated in an alcohol gradient. The procedures going forward for tissue slides were the same as those of TP slides. All slides were counterstained with hematoxylin and mounted for microscopic examination. The known positive tissue samples (breast) of patients were used as positive controls, and the PBS, as the substitute for the primary antibody, were used as the negative controls.

Nuclear staining of the cancer cells was regarded as positive according to the percentage of stained cells seen on cytological and histological slides. The cutoff value was 1% in terms of the ASCO/CAP guidelines for HRs of breast cancer and each sample included a negative control antibody.[9]

HER2 status was evaluated by ICC in terms of the ASCO/CAP guidelines.[10] In brief, those samples, whose membrane has no staining or partial staining, which is not complete and faintly/barely observed in $\leq$10% of tumor cells, were defined as 0; partial membrane staining, which is faintly/barely observed in $>$10% of tumor cells, was defined as 1+; circular membrane staining, which is not complete and/or weak/moderate in $>$10% of tumor cells, or complete and circular membrane staining, which is intense in $\leq$10% of tumor cells, were defined as 2+; circular membrane staining, which is complete, intense in $>$10% of tumor cells, were defined as 3+. Those samples were regarded as negative if their staining scores were 0 and 1+. The samples with staining scores of 2+ and 3+ were regarded as equivocal and positive, respectively. All cases were observed blindly and independently by 2 pathologists; discrepant results were resolved by joint review. Additional FISH tests were performed on immune-staining cases scored as 2+, for the purpose of evaluating the gene status.

FISH Analysis for HER2 Amplification

The commonly used dual-probe PathVysion kit (Abbott Molecular, Des Plaines, IL) was chosen to assess *HER2* gene status on FFPE samples and TP preparations, which are performed by similar procedures. But FNAC TP preparations were no longer necessary for deparaffinization and the initial hydrochloric acid treatment. After counterstained with 4,6-diamidino-2-phenylindole (DAPI), all slides were mounted for microscopic examination. In order to calculate the ratio of orange signals (the *HER2* gene)/green signals (chromosome enumeration probe 17, CEP-17), all samples were observed by means of fluorescence microscope (Olympus U-TV0.63×C 9A05367, Japan; 1000× magnification) with 3 filters DAPI, Spectrum Orange, and Spectrum Green.

HER2 gene amplification status was determined based on the ASCO/CAP guidelines.[10] In each sample, 20 cells were counted and the ratio of *HER2* to CEP17 was calculated. The *HER2* gene was considered amplified if the *HER2*/CEP17 ratio $\geq$2.0 or *HER2*/CEP17 ratio $<$2.0 but average *HER2* copy number $\geq$6.0 per cell. The *HER2* gene status was considered equivocal if the *HER2*/CEP17 ratio $<$2.0 but average *HER2* copy number $\geq$4.0 while $<$6.0 per cell. The *HER2* gene was considered not amplified if the *HER2*/CEP17 ratio $<$2.0 and average *HER2* copy number $<$4.0 per cell. All cases were evaluated blindly and independently by 2 pathologists; discrepant results were reconsidered by joint review.

Statistical Analysis

The HRs and HER2 overexpression was detected on the cytological and histological specimens for each case. The McNemar test was applied to assess whether similar results were generated by the 2 methods. If the obtained *P* values were less than 0.05, they were regarded as significant.

The Cohen kappa statistic was utilized to assess the agreement between the cytological and histological slides. Kappa values $>$0.6 mean that the agreement was good. Kappa values $>$0.8 mean that the agreement was very good. In the procedure of analysis, only the cases in which the status of the considered markers was capable of being assessed in both by TP preparations and the matched tissue samples were evaluated. All statistical analyses were carried out using the Statistical Analysis System.

RESULTS

In this study, all enrolled patients were women ranging in age from 26 to 95 years (mean, 53 years). The majority (97.9%) had invasive ductal carcinoma; the most frequent histological grades were 2 and 3. The amount of ductal cancer in situ (DCIS) was observed on the tissue slides, DCIS components were not observed in 499 samples of the total. The remaining 43 samples

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TABLE 1. Pathologic and Clinical Characteristics of the Cases

Characteristic	No. of Cases or Patients, %
Histotype	
Invasive ductal carcinoma	537 (99.1)
Invasive mucinous carcinoma	5 (0.9)
Ductal carcinoma in situ	
Absent	499 (92.1)
<25%	30 (5.5)
>25%	13 (2.4)
Tumor diameter	
>2 cm	417 (76.9)
<2 cm	125 (23.1)
Lymph node	
Positive	249 (45.9)
Negative	293 (54.1)

that were diagnosed as invasive ductal carcinoma showed in situ components. Thirty cases comprised a DCIS component of less than 25% in each case of lesion. Only 13 samples were found comprising DCIS component larger than 25% in each case of lesion. The majority of the cases showed with tumors having >2 centimeters (76.9%) of their maximum diameter, and 249 (45.9%) cases were positive for lymph node metastases. Meanwhile, 43 out of 542 cases (7.9%) were diagnosed as invasive ductal carcinoma which showed in situ component. Thus, we always obtain samples in the middle of the tumor masses, avoiding peripheral DCIS area (see Table 1).

The HRs and HER2 status of all 542 FFPE samples and the corresponding satisfactory TP specimens were assessed by IHC and ICC, respectively (Table 2). Among the 542 TP specimens, the slides with cell number ≥100 were considered satisfactory and included in this analysis. In total, 520 slides for ER, 517 slides for PR, and 529 slides for HER2 status analysis were included in this analysis (Table 2). As shown in Table 3, the positive rates of ER in TP and FFPE specimens were 63.2% and 63.7%, respectively ($P = 0.8455$); and those of PR were 65.9% and 66.2%, respectively ($P = 0.8712$). The positive rates of HER2 were 39.7% and 56.1%, respectively ($P < 0.0001$), which was statistically significant.

ER Status

Total 520 pairs of samples possess results of ER expression for both TP slides and corresponding FFPE tissue specimens (see Figure 1A, B). Twenty two TP specimens were not assessable for ER expression due to unsatisfactory quality (Table 2). For the 485 of the 520 samples, our evaluation results of the TP-processed samples correlated well (concordance rate = 93.3%, kappa = 0.85) with those of the tissue slides. The sensitivity of ER assessment was 94.7%, and the specificity was 90.7%. There were 35 discordant results. The differences of the assessment of the ER overexpression between the 2 methods were not statistically significant ($P = 0.8618$) (Table 4).

TABLE 2. Quality of Thin-Prep Slides

TP	ER, %	PR, %	HER2, %
Cell numbers ≥ 100	520 (95.9)	517 (95.4)	529 (97.6)
Cell numbers < 100	16 (3.0)	18 (3.3)	8 (1.5)
Slipped slides	6 (1.1)	7 (1.3)	5 (0.9)
Total	542	542	542

ER = estrogen receptor, HER2 = human epidermal growth factor receptor 2, PR = progesterone receptor, TP = Thin-Prep.

TABLE 3. Immunohistochemical Results of Hormone Receptors and Human Epidermal Growth Factor Receptor 2 Status in Thin-Prep Preparations and Formalin-Fixed Paraffin-Embedded Tissue Samples

		TP	FFPE	χ^2	*P*
ER, %				0.0380	0.8455
	Positive	328 (63.2)	345 (63.7)		
	Negative	192 (36.8)	197 (36.3)		
	Total	520	542		
PR, %				0.0263	0.8712
	Positive	340 (65.9)	359 (66.2)		
	Negative	177 (34.1)	183 (33.8)		
	Total	517	542		
HER2, %				17.3787	<0.0001
	3+	96 (18.1)	129 (23.8)		
	2+	114 (21.6)	175 (32.3)		
	1+/0	319 (60.3)	238 (43.9)		
	Total	529	542		

ER = estrogen receptor, FFPE = formalin-fixed paraffin-embedded, HER2 = human epidermal growth factor receptor 2, PR = progesterone receptor, TP = Thin-Prep.

PR Status

Among the 542 TP specimens, 25 cases were not assessable (Table 2). There were total 517 pairs of samples had PR status results for both TP and corresponding FFPE tissue specimens (see Figure 1C, D). For the 458 of the 517 samples, our evaluation results of the TP-processed samples correlated well (concordance rate = 88.6%, kappa = 0.75) with those of the tissue slides. The sensitivity of PR status was 91.4%, and the specificity was 83.1%. There were 59 discordant results. The differences of the assessment of the PR overexpression between the 2 methods were not statistically significant ($P = 0.8907$) (Table 5).

HER2 Status

For HER2 status assessment, 13 cases were not assessable from TP specimens (Table 2). So, there were 529 pairs of samples that had results for both FNAC and the tissue samples (see Figure 1E, F). The evaluation results of TP preparation correlated well with those of the tissue slides in 423 of the 529 samples (concordance rate = 80.0%, kappa = 0.62) (Table 6). The agreement cases in positive expression between 2 methods were 204. One hundred fourteen cases express a score of 2+ in TP specimens. The HER2 sensitivity and specificity were 67.1% and 97.3%, respectively. There were 106 discordant results. The positive predictive value was 97.1%, and the negative predictive value was 68.8%. The differences observed between the 2 methods when used to evaluate HER2 status were statistically significant ($P < 0.0001$).

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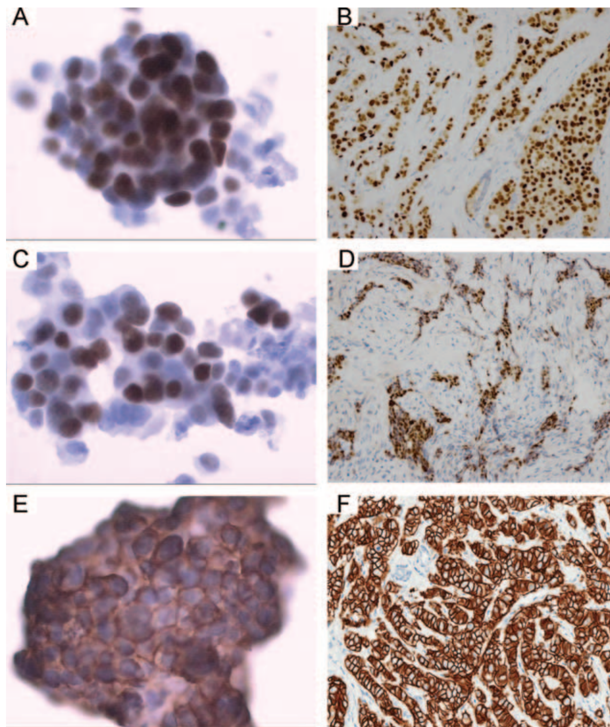

FIGURE 1. Immunohistochemistry is shown for estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) on the cytologic Thin-Prep (TP) slides and on the corresponding histologic sections (A–F, DAB staining). ER immune-staining is shown on a TP cytologic slide (panel A, ×400, and on the corresponding histologic section (panel B, ×200) in breast cancer; a strong positive reaction for ER is seen on the nuclei of the cancer cells in both specimens. PR immune-staining is shown on a TP cytologic slide (panel C, ×400) and on the corresponding histologic section (panel D, ×200) in breast cancer; a strong positive reaction for PR is seen on the nuclei of the cancer cells in both specimens. HER2 immune-staining is shown on a TP breast carcinoma cytologic slide (panel E, ×400) and on the corresponding histologic section (panel F, ×200); a strong complete membrane staining for HER2 (score 3+) is observed in both specimens.

HER2 FISH Results

Among the 529 FNAC TP samples with HER2 ICC results, 114 were scored 2+. Performing FISH assay with these ICC equivocal cases, we found that 41.2% (47/114) samples were *HER2*-amplified. FISH assay results of the corresponding FFPE samples revealed that 40.4% (46/114) were *HER2*-amplified (Figure 2A, B). Of these 114 pairs of specimens, 113 pairs showed concordant results (kappa = 0.98, $P = 0.3173$), showing high concordant rate between FNAC and FFPE FISH results (99.1%). In the discordant pair, FISH of the FFPE specimen indicated negative result for *HER2* gene amplification, while that of the FNAC specimen was positive (*HER2*/CEP-17 = 1.3 vs *HER2*/CEP-17 = 4.2). Repeating FISH for this case showed similar result (*HER2*/CEP-17 = 1.5 in FFPE samples, *HER2*/CEP-17 = 3.8 in FNAC specimens) (Figure 3A, B, Table 7).

TABLE 4. Estrogen Receptor Status in Thin-Prep Preparations and the Corresponding Formalin-Fixed Paraffin-Embedded Tissue Specimens

	ER Status (FFPE)		
ER Status (TP)	Positive	Negative	Total
Positive	318	17	335
Negative	18	167	185
Total	336	184	520

ER = estrogen receptor, FFPE = formalin-fixed paraffin-embedded, TP = Thin-Prep.

TABLE 5. Progesterone Receptor Status in Thin-Prep Preparations and in the Corresponding Formalin-Fixed Paraffin-Embedded Tissue Specimens

	PR Status (FFPE)		
PR Status (TP)	Positive	Negative	Total
Positive	310	30	340
Negative	29	148	177
Total	339	178	517

FFPE = formalin-fixed paraffin-embedded, PR = progesterone receptor, TP = Thin-Prep.

DISCUSSION

As an accurate and sensitive modality for obtaining diagnosis samples, FNAC has been increasingly used as a means in the diagnosis of breast cancer. The tissue collected is used for diagnostic purposes as well as for a multitude of ancillary tests including prognostic and predictive biomarkers. FNAC is a valuable alternative method to obtain specimens for diagnosis and evaluation of HRs and HER2 status and *HER2* gene amplification. This method may be particularly beneficial for patients with lesions that are not amenable to excisional/core biopsies or for patients with inoperable or metastatic BC. Idirisinghe et al[13] reported that different overexpression in HRs and HER2 statue were observed in primary and metastatic cancer cells. Therefore, these biomarkers should be reevaluated before patients get further treatment. For recurrence and metastatic tumors, FNAC may be the optimal choice for obtaining tissue material since the operation is simpler and has relatively lower risk of complications than traditional tissue biopsies.[14]

The cytology specimens prepared by the TP method can be preserved for several months or even years. TP slides usually

TABLE 6. Human Epidermal Growth Factor Receptor 2 Status in Thin-Prep Preparations and in the Corresponding Formalin-Fixed Paraffin-Embedded Tissue Specimens

	HER2 Status (FFPE)		
HER2 Status (TP)	Positive	Negative	Total
Positive	204	6	210
Negative	100	219	319
Total	304	225	529

FFPE = formalin-fixed paraffin-embedded, HER2 = human epidermal growth factor receptor 2, TP = Thin-Prep.

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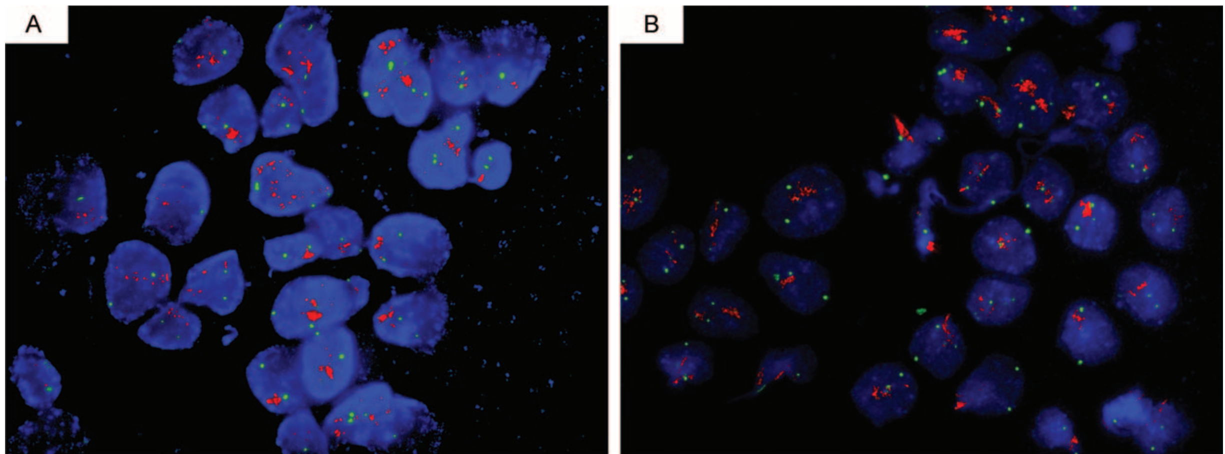

FIGURE 2. Fluorescence in situ hybridization (FISH) is shown for HER2 gene amplification on Thin-Prep (TP) specimens and on the corresponding histologic sections (A, B, DAPI staining). (A) HER2 gene amplification is shown on a TP cytology specimen (panel A, ×1000, FISH ratio, HER2/CEP-17 = 3.9). (B) HER2 gene amplification is shown on the corresponding histologic section (panel B, ×1000, FISH ratio, HER2/CEP-17 = 4.2).

have a clear background, with less nonspecific staining and higher cell density, thus minimizing the antibodies used. Smears processed with the TP method provide high quality staining of cells which are easily assessed.[15,16] Furthermore, several antibodies can be used on the same slide simultaneously, enhancing the efficiency of molecular diagnosis. Some latest reports about HRs protein overexpression and *HER2* gene amplification assessment for breast cancer by ICC and FISH using FNAC specimens showed excellent concordance rates between FNAC slides and FFPE sections.[17–24] The use of FNAC specimens in determining the HRs status and the level of *HER2* gene amplification can achieve high sensitivity, accuracy, and reliability. In this study, we used diagnostic results of HRs and HER2 status of FFPE tissue specimens as the gold standard to investigate if molecular diagnosis with TP-processed specimens is applicable to clinical practice.

Our immune-staining data show very good concordance rates (ER status: 93.3%, PR status: 88.6%) and excellent consistency (ER status: kappa = 0.85, PR status: kappa = 0.75) 0.75) by the Cohen kappa test between FNAC slides and the corresponding tissue sections, and no any statistically significant differences were found between the 2 methods by McNemar test (ER status: $P = 0.8618$, PR status: $P = 0.8907$). These results are consistent with the findings in previous reports.[17,18,24,25] Enrico et al evaluated the ER and PR status in 111 patients with BC by 2 methods, reporting concordance rates of 98.0% (kappa = 0.92) and 90.0% (kappa = 0.76), respectively. Our evaluation results were in line with those above that the assessment of HRs protein overexpression for TP specimens is basically at the same reliable level as tissue sections.

The reason for the false negative results upon rereading the TP slides is that although a small number of cells are positive in the slides, they are interpreted as negative due to their intermediate intensity. The reason for false positive results is that in some slides, both the cytoplasm and nuclei are stained. Thus, these slides are mistakenly judged as positive. Additionally, the heterogeneity of positive cells in the tumor is also one of the key

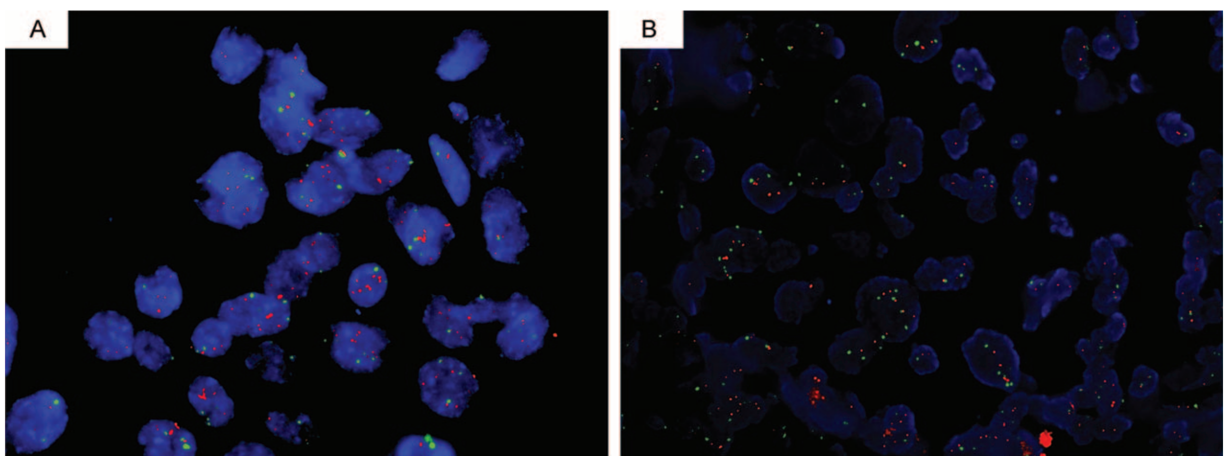

FIGURE 3. A and B were showed for the same case (A, B, DAPI staining). Fluorescence in situ hybridization (FISH) on a Thin-Prep cytology specimen indicated positive result for HER2 gene amplification, while the same case histologic specimen indicated negative result. (panel A, B, ×1000, HER2/CEP-17 = 3.8, HER2/CEP-17 = 1.5).

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TABLE 7. Comparison of Fluorescence In Situ Hybridization Results From 114 Immunocytochemistry Equivocal Fine Needle Aspiration Cytology Specimens and the Corresponding Formalin-Fixed Paraffin-Embedded Specimens

HER2 FISH (FNAC)	*HER2* FISH (FFPE) Amplification	No Amplification	Total
Amplification	46	1	47
No amplification	0	67	67
Total cases	46	68	114

FFPE = formalin-fixed paraffin-embedded, FISH = fluorescence in situ hybridization, FNAC = fine needle aspiration cytology, HER2 = human epidermal growth factor receptor 2.

causes for inconsistencies between cytology and histology results.[26,27]

HER2 is an oncogene. HER2 protein overexpression and gene amplification are associated with poor prognosis in breast cancer. In patients with HER2-positive breast cancer, the tumor is normally more invasive, leading to worse prognosis and shorter survival time.[28] Overexpression of HER2 protein and amplification of *HER2* gene are the major predictive biomarkers for trastuzumab therapy. The HER2 diagnostic test has been utilized and standardized for FFPE tissue samples, having a positive rate of HER2 protein expression of 10%–35% in FFPE tissue samples.[19,20,29,30] Nevertheless, FFPE preparations may not be a feasible option for pathological evaluation for patients who are not suitable for surgery. Recent studies have reported results of HER2 status assessment by using FNAC breast cancer specimens (CB or TP) to evaluate HER2 protein status by ICC. The concordance rate between the HER2 protein overexpression rates of CB and TP-processed FNAC specimens was 79.0%–95.2%; the sensitivity was 70.0%–84.0%, and the specificity was 87.9%–100.0%.[30–33] Bedard et al reported there was a concordance rate of 78% between the cytological and histological samples. Seventy one percent cases expressed positive results on TP specimens, while presenting a negative result on the matched tissue samples. Therefore, the authors made a conclusion that negative ICC HER2 result was reliable. However, they believed the positive result was unreliable and should be confirmed by FISH assessment. In another study, Beatty et al[19] assessed HER2 protein expression and gene amplification in 51 FNAC samples and the matched FFPE specimens. In addition, they evaluated 3 fixation methods with the cytology samples, including ethanol, formalin, and TP. Both the TP and the formalin method demonstrated a moderate (k = 0.692 and 0.667) agreement between FNAC ICC and tissue IHC results. The authors concluded that HER2 overexpression from TP was unreliable for clinical use. The HER2 status, detected by ICC and IHC, was moderately consistent in most reports.[19,30–33] However, in the Enrico et al's study, the authors found perfect agreement of HER2 status, a 100% concordance rate (kappa = 1.0), between TP and FFPE specimens. Thus, they concluded that TP is valuable for detecting HER2 status in IBC, especially for metastatic lesions. And they believed the inconsistency of HER2 ICC evaluation results could arise from the minor difference of laboratory conditions and procedures. They concluded that the reasons for low sensitivity were delayed fixation and ICC assessment.[24]

In this study, we collected and assessed samples from 542 patients with BC. Our study showed that HER2 status immune-staining results of the FNAC samples were concordant with those of the tissue sections in 423 of the 529 assessable samples (concordance rate = 80.0%, kappa = 0.62). There were 106 discordant results (Table 6). Our HER2 detection for both FNAC ICC and tissue IHC demonstrated a moderate consistency, which is consistent to most previous studies.[19,30–33] In 106 discordant cases, 100 (96.2%) were false negative (ICC negative in FNAC specimens, IHC positive in the matched FFPE specimens). This was mainly because small number of positive cells was interpreted as negative due to intermediate intensity of staining. In addition, the sensitivity of FNAC HER2 ICC was 67.1%, and the negative predictive value was 68.8%. Enrico et al[24] believed that delayed fixation and ICC assessment of the samples were the reasons for the low sensitivity. Although these 2 potential factors were avoided in our study, the concordance rate was only 80.0%. Different detection technique and procedure used in this study together with the heterogeneity of tumor cells may be the reasons of this inconsistency.[26,27] In contrary to Bedard et al,[33] the specificity of FNAC HER2 ICC in our study was very high (97.3%), and the positive predictive value was 97.1%. It is worth to note that all FNAC specimens were votexed for 20 to 30 minutes before slide preparation in our laboratory, this might explain a relatively high specificity of HER2 ICC in this study. Determining and assessing complete membrane positivity in 3-dimensional clusters can be difficult. One approach may be to votex FNAC specimens in CytoLyt solution for 20 to 30 minutes to reduce 3-dimensional clusters before slide preparation. However, we do not have direct evidence to support this notion. Further studies are required to investigate if votexing actually improves accuracy of TP materials.

In this study, 114 HER2 ICC 2+ equivocal cases were subjected to further FISH assay comparison for both TP specimens and corresponding FFPE specimens. The concordance rate of FISH results of *HER2* gene amplification was 99.1% (kappa = 0.98, $P = 0.3173$), which is consistent with previous studies (98%–100%).[19–21] Of the only pair of specimens with discordant result, the FFPE specimen was scored 2+ by IHC and diagnosed as negative for *HER2* gene amplification by FISH (*HER2*/CEP-17 = 1.3), while the corresponding FNAC specimen was diagnosed as *HER2* gene amplified by FISH (*HER2*/CEP-17 = 4.2). Histology test confirmed this case as a grade II infiltrating ductal carcinoma containing no in situ components. Since FISH is conducted on whole nuclei of FNAC specimens and truncation artifacts are avoided, it gives the most accurate assessments of gene and chromosome copy numbers, resulting in accurate gene/chromosome ratios. Nevertheless, the heterogeneity of cancer cells (various amplification ratios in different cells of the same tumor) may be a reason for this observation.[34–36] Moreover, the results reported by Enrico et al[24] showed that the shorter fixation time may influence HER2 results. It is worth noting that our specimens for *HER2* gene amplification detection were preserved for 0.5 to 27 months, and results were not affected by the long storage time. This implied that TP-processed FNAC specimens could be stored for 2 years without substantially affecting results of FISH analysis.

Here, we performed assessment of HR levels and HER2 protein expression in 542 TP-processed FNAC breast cancer specimens. The results of HR levels using FNAC specimens correlated well with those of FFPE specimens, with high sensitivity. HER2 ICC assessment using TP-processed FNAC specimens had lower sensitivity but high specificity, indicating that the positive results of HRs and HER2 ICC in FNAC

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specimens were reliable for clinical diagnosis. Negative results of ICC, especially for HER2, in FNAC specimens were unreliable. However, FISH analysis for the 114 equivocal cases for HER2 ICC with TP-processed slides possesses high concordance rate with FFPE samples.

In conclusion, in the patients whose histology specimens are not easy to obtain or when recurrence is present, the results of ICC HRs in TP FNAC specimens may be reliable, but HER2 assessment is not reliable enough for clinical use. FISH testing is necessary in this setting.

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