HER 2 Immunohistochemistry for Breast Cancer Cell Blocks Can Be Used in the Same Way as That Used for Histological Specimens

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Background: *Human epidermal growth factor receptor 2 (HER2) testing of samples from recurrent or metastatic breast cancer is recommended by the 2013 update of the American Society of Clinical Oncology/College of American Pathologists guidelines. Although cytological analysis can be applied to several types of metastatic lesions, the practical method for HER2 testing of cytological specimens is yet to be resolved. We conducted immunohistochemical (IHC) staining for HER2 in breast cancer cell blocks (CBs) and compared the results with those from the corresponding histological specimens. In cases of discrepancy between the two types of specimen, the bright-field HER2 dual in situ hybridization (DISH) assay was performed.*
Methods: *CBs were prepared from 54 surgically excised breast cancers. The cells were fixed in 10% buffered formalin and embedded in paraffin. A Ventana BenchMark ULTRA (Roche Diagnostics) with anti-HER-2/neu (4B5) rabbit monoclonal primary antibody and INFORM HER2/neu Dual ISH DNA Probe Cocktail was used for the assays.*
Results: *Successful results were obtained in 52 of 54 CBs. Forty cases showed agreement between CBs and the histological specimens. No discrepancy was observed between the two types of specimens in cases where HER2 expression was positive. IHC results of CB in 12 discrepant cases were HER2 intermediate or negative. The DISH results of 11 of these cases were negative.*
Conclusion: *IHC staining of HER2 for breast cancer CBs can be used in the same way as that used for histological specimens, although the number of equivocal cases in CBs is greater than that in histological specimens.* Diagn. Cytopathol. 2016;44:274–279. © 2016 The Authors Diagnostic Cytopathology Published by Wiley Periodicals, Inc.

Key Words: breast cancer; cell block; HER2; immunohistochemistry; DISH

Human epidermal growth factor receptor 2 (HER2) testing of samples from recurrent or metastatic breast cancers is recommended by the 2013 update of the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines[1] because of potential differences in HER2 status between primary and metastatic breast cancer sites. Cytological analysis can be applied to several types of metastatic lesion as well as to body cavity fluids, and it is a useful approach for patients in poor clinical condition.

There are issues regarding HER2 testing for cytological specimens that remain to be resolved, although some studies comparing the HER2 status of cytological specimens from metastatic sites with that from histological results of the primary tumor site have been conducted.[2,3] The HER2 gene amplification visualization in cytological specimens using fluorescence in situ hybridization (FISH) demonstrates a strong and consistent correlation with the HER2 status of the tissue samples.[4–7]. However, there are limitations to the FISH assay, such as the need of dark-field fluorescence microscopy and the inability to

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visualize the morphological details. To overcome some of these limitations, the bright-field HER2 dual in situ hybridization (DISH) assay was developed.[8–10] Although we believe that the bright-field HER2 DISH assay can be a reliable and practical method to assess the HER2 status of breast cancer cytological specimens in routine clinical practice, there are also limitations to the DISH assay, such as the need for a dedicated autostainer, expensive reagents, and the time required for counting signals.

Several studies cite that immunohistochemical (IHC) detection of HER2 protein expression in cytological specimens is unreliable because of unstable staining.[4,5,11–15] However, one study has reported that formalin-fixed cell blocks (CBs) from primary or metastatic breast carcinoma showed 100% correlation with their corresponding histological sections[3] and the other study has reported that CBs are reliable for IHC detection of HER2.[16]

Here, we conducted HER2 IHC staining on CBs prepared from cancer cell samples collected from surgically excised breast cancers. We compared the obtained results to those from the corresponding histological specimens to assess the reliability of the HER2 IHC method performed on CBs. In addition, where there was discrepancy between the results obtained from CBs and the corresponding histological specimens, the HER2 DISH assay was performed.

Materials and Methods

This study was conducted under the Ethical Guidelines for Medical and Health Research Involving Human Subjects by the Health, Labor and Welfare Ministry of Japan (http://www.mhlw.go.jp/stf/seisakunitsuite/bunya/hokabunya/kenkyujigyou/i-kenkyu/). According to Number 6 (Omission of procedures concerning informed consent, etc.), Chapter 5, Part [12] in this guideline, informed consent was not obtained from the subjects.

CBs were prepared from tumor cell samples collected from 54 surgically excised breast tumors. CBs and the corresponding histological specimens were cut to ~4 μm thickness and were prepared on silanized glass slides. IHC staining and the DISH assay were then performed for both CB and histological specimen sections. The staining and assay were performed with a Ventana BenchMark ULTRA (Roche Diagnostics, Basel, Switzerland). The 2013 ASCO/CAP criteria for HER2 testing in breast cancer[1] was used to categorize the results. Fifty-two out of 54 cases were analyzed. Two cases were not used because of low numbers of cells on the CB slides. HER2 expression on CBs and that on the corresponding histological specimens was compared. The HER2 DISH assay was performed where there was discrepancy between the IHC results of HER2 obtained from CBs and from the corresponding histological specimens.

Preparation of CBs

A single specimen was collected from each tumor using a 21-gauge needle attached to a 20 ml syringe mounted on an aspiration gun. The cells were fixed in 10% buffered formalin for 16–28 hour, processed for CB preparation by the sodium alginate method, and embedded in paraffin. The CB preparation was performed as follows: sample-containing tubes were centrifuged at 3000 rpm for 5 min, formalin was removed, 1% sodium alginate was added, tubes were centrifuged at 3000 rpm for 5 min, and 1*M* calcium chloride was added. The gel pellet formed by this process was used as the histological specimen.

Preparation of Histological Specimens

Representative sections were prepared from the cut surface of the resected breast tumors. Tissues were fixed in 10% buffered formalin for 24–48 hour and embedded in paraffin.

Histological Breast Cancer Types

The following tumors were included: 49 invasive ductal carcinomas of no special type, two invasive lobular carcinomas, two noninvasive ductal carcinomas, and one mucinous carcinoma.

IHC Staining and Evaluation of the Results

IHC staining was performed on both the CB and histological sections using the Ventana iVIEW DAB Detection Kit. The staining procedure using this kit is based on the indirect biotin streptavidin system. The protocol involving heat antigen retrieval was used as recommended by the manufacturer for paraffin-embedded sections. For the primary antibody, the anti-HER-2/neu (4B5) rabbit monoclonal primary antibody of Ventana I-VIEW PATHWAY (Roche Diagnostics) was used.

Staining results were scored as 0, 1+, 2+, or 3+ according to the following criteria: strong circumferential membranous staining in >10% of tumor cells was considered as 3+; moderate circumferential staining in >10% of tumor cells or strong circumferential membranous staining in ≤10% of tumor cells was considered as 2+; weak and incomplete membranous staining in >10% of tumor cells was considered as 1+; and the absence of staining or weak and incomplete membranous staining in ≤10% of tumor cells was considered as 0. The HER2 expression was considered as negative if scored as 0 or 1+, intermediate if scored as 2+, and positive if scored as 3+.

DISH Assay and Evaluation of the Results

The INFORM *HER2*/*neu* Dual ISH DNA Probe Cocktail assay was performed on both the CB and tissue sections. The DISH assay was performed according to the manufacturer's recommended protocol for surgical specimens. The standard protocol was initially performed for both types of sections; however, the protease reaction time was extended if signals were weak. The *HER2*/*neu* (black) to chromosome enumeration probe 17 (CEP17) (red) ratio was manually counted using a light microscope in each specimen by one investigator to avoid subjective bias, and the result was confirmed by a second investigator. At least 20 cells were counted.

The criteria consist of a combination of the HER2/CEP17 ratio and the average number of HER2 signals per cell. The HER2 gene amplification was scored as "amplified" if the case had a HER2/CEP17 signal count ratio of 2.0 or if the HER2/CEP17 signal count ratio was <2.0 but the average number of HER2 signals per cell was 6.0. A score of "equivocal" was given if the case had a HER2/CEP17 signal count ratio of <2.0 and the average number of HER2 signals per cell was ≥ 4.0 and <6.0. A score of "not amplified" was given if the case had a HER2/CEP17 signal count ratio of <2.0 and the average number of HER2 signals was <4.0. CB results were compared with the tissue results from the same case.

Table I. Comparison of HER2 Expression in CBs and Histological Sections

	Histological section			
CB	*Negative*	*Intermediate*	*Positive*	*Total*
Negative	20	2	0	22
Intermediate	10	9	0	19
Positive	0	0	11	11
Total	30	11	11	52

HER2, human epidermal growth factor receptor 2.

Data Management

The Fleiss–Cohen's weighted kappa coefficient was used to assess the correlation between the results from CBs and those from the tissue specimens. The correlation was scored as "good" if the kappa-value exceeded 0.6 and "excellent" if it exceeded 0.8.

The weighted kappa coefficient was calculated by Microsoft Office Excel 2013 software.

Results

Comparison of HER2 Expression in CBs and Histological Specimens

Of the 52 cases, 40 cases showed agreement between CBs and corresponding histological sections (concordance rate, 77%; weighted kappa, 0.818) (Table I). Twenty cases were HER2 negative (Fig. 1), 9 were intermediate, and 11 were positive (Fig. 2). There were 12 discrepant cases. Of these, 10 cases were HER2 intermediate in CBs but negative in histological sections, and two cases were HER2 negative in CBs but intermediate in histological sections. No discrepancy between the two types of specimen was observed in cases where HER2 expression was positive.

HER2 DISH Results of Discrepant Cases Between CBs and Histological Specimens

The HER2 DISH results of the 10 cases where intermediate HER2 expression was observed in the CB sections but was negative in histological sections are shown in Table II. Eight cases were not amplified by HER2 DISH in both histological specimens and CBs (Fig. 3). One case was not amplified for the histological specimen but amplified for the CB. The HER2/CEP17 signal count ratio of this case was 1.3 in the histological specimen and 2.0 in the CB. The weakness of CEP17 signals in the CB of this case leads to underestimation of the true CEP17 signal count. This case should be categorized as an unamplified

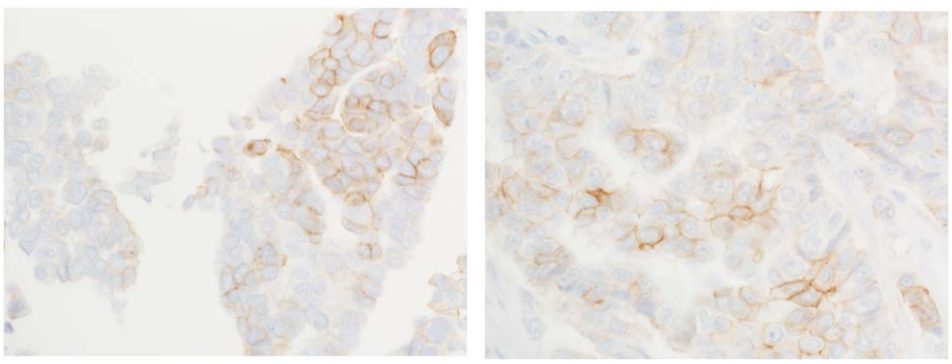

Fig. 1. An example of a HER2 negative (1+) case showing consistent results between the CB (**a**) and histological specimen (**b**). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

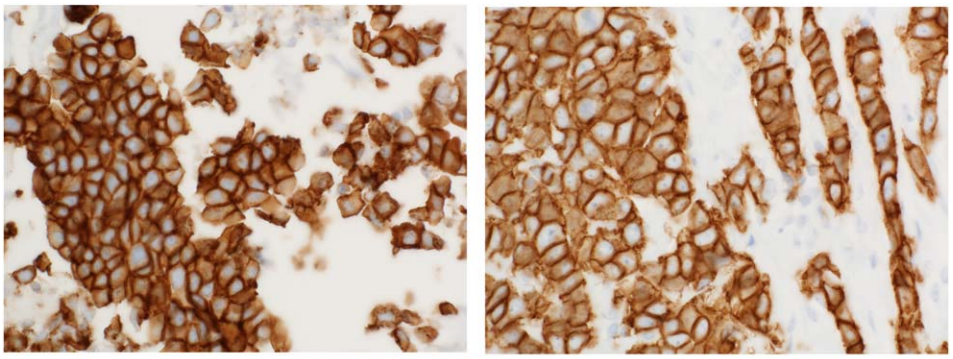

Fig. 2. An example of a HER2 positive (3+) case showing consistent results between the CB (**a**) and histological specimen (**b**). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table II. HER2 DISH Results for Cases of Intermediate HER2 Expression on CBs but Negative HER2 on Histological Section

HER2 DISH results		
Histological section	*CB*	*No. of cases*
Not Amplified	Not Amplified	8
Not Amplified	Amplified	1
Equivocal	Amplified	1
Total		10

HER2, human epidermal growth factor receptor 2; DISH, dual in situ hybridization.

case by HER2 DISH. The last of the 10 discrepant cases was observed as "equivocal" in the histological specimen but as "amplified" in the CB. The HER2/CEP17 signal count ratio of this case was 1.5 in the histological specimen and 2.2 in the CB. The average number of HER2 signals on the histological section was 4.2 per cell.

Of the two cases showing HER2 negative in CBs but HER2 intermediate in histological specimens, one case was not amplified by HER2 DISH in both histological specimen and CB, and one case was amplified on the histological section but not amplified on the CB section. The HER2/CEP17 signal count ratio of this latter case was 2.0 in the histological specimen and 1.4 in the CB.

Discussion

Evaluation of the HER2 receptor status at metastatic sites is important for selecting the correct chemotherapy for treatment of recurrent disease.[1] Cytology can be applied to several types of metastatic lesion from which biopsies may be difficult to obtain, a particular example being from body cavity fluids. Moreover, cytology is useful for patients in poor clinical condition. The use of cytology can therefore be a rapid, inexpensive, and less traumatic alternative to biopsy in these situations.

Several studies have reported that hormone receptor status, monitored in various types of cytological specimens, correlates well with the corresponding histological specimen.[17–19] However, there are issues that remain to be resolved regarding HER2 testing for cytological specimens before this method can be adopted in routine clinical practice.

Although the FISH assay of cytological specimens demonstrates strong and consistent correlation with the HER2 status of original tissue samples,[4–7] this method has some disadvantages for clinical use. In the FISH assay, morphological features are difficult to visualize under dark-field imaging. Therefore, the FISH assay is not suitable for cytological specimens taken from body cavity fluids or aspirates because of the presence of non-neoplastic inflammatory cells. In addition, fluorescence fades quickly, thus FISH is unable to provide a durable record.

The DISH assay could be a reliable and practical method to assess the HER2 status of breast cancer cytological specimens. Several studies have utilized the HER2 DISH assay on liquid-based cytology specimens, using the ThinPrep technique[8,20,21] and CBs.[9,10,22] We therefore used the DISH assay to assess the reliability of discrepant cases regarding IHC results between CBs and corresponding histological specimens.

False positive results of IHC staining for HER2 on alcohol-fixed cytological specimens are a major problem in smears[8] and CBs.[14,15] In a preliminary study at our institution, we encountered difficulties in scoring the HER2 status by IHC methods using liquid-based cytological specimens stored in ThinPrep PreservCyt Solution (Hologic) and found a low concordance rate with the corresponding histological specimens (data not shown). According to the reasoning mentioned above, formalin-fixed CBs are recommended for HER2 IHC staining.[2,3] However, discordant results are reported between formalin-fixed CBs and tissue specimens. One reason is the errors of interpretation on CB analysis, potentially resulting from abundant cytoplasmic as well as background staining.[12] Also, it appears that HER2 expression can be overestimated in small biopsy samples.[2,13]

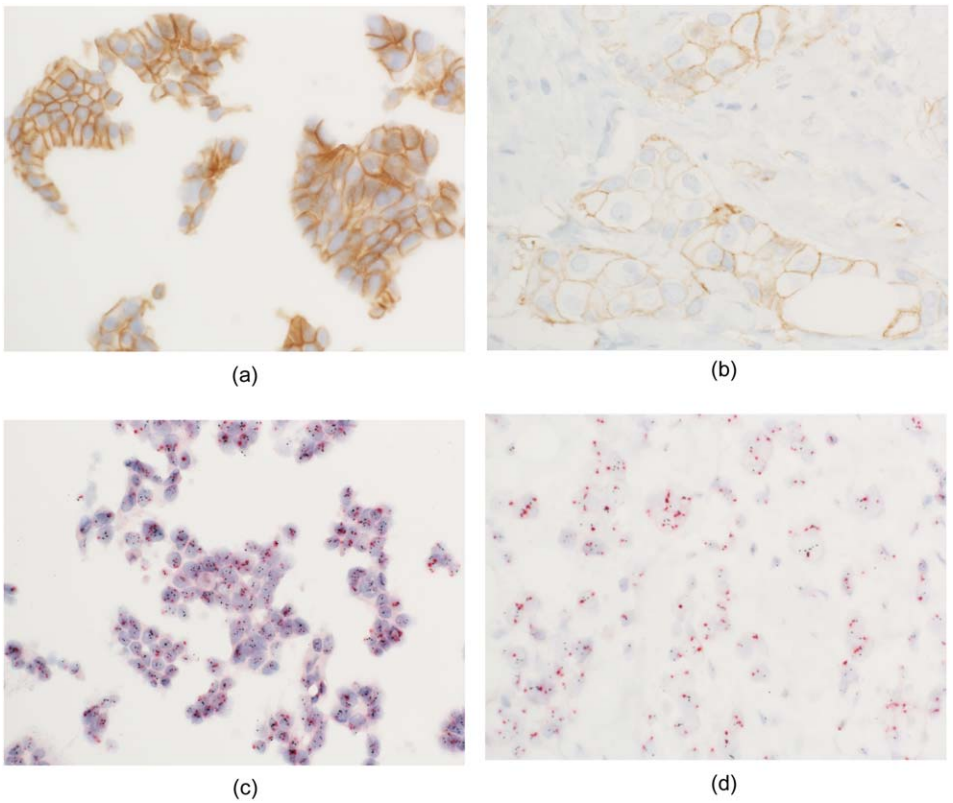

Fig. 3. An example of a discrepant case of HER2 expression, showing HER2 intermediate (2+) in the CB (**a**) and HER2 negative (1+) in the histological specimen (**b**). The results by DISH assay in both CB (**c**) and histology are negative (**d**). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Although our study showed excellent agreement between CBs and corresponding histological specimens (weighted kappa, 0.818), there were 12 discrepant cases. The IHC results of CBs in these 12 discrepant cases were HER2 intermediate (2+) or HER2 negative (0 or 1+). There were no discrepant cases among the HER2 positive results (3+).

The HER2 DISH assay was used to confirm the results in the cases where HER2 expression was intermediate on CBs but negative on histological sections. Eight of 10 cases were not amplified by HER2 DISH in both histological specimens and CBs. These eight cases should therefore be categorized as negative. One case that was unamplified on the histological section but was amplified on the CB section should be categorized as an unamplified case because the discrepancy was caused by the weakness of the CEP17 signals on the CB section. The cause of discrepancy of the other one case was thought to be caused by the distribution of amplified cells. Therefore, these cases with intermediate HER2 expression by IHC staining on CBs but negative expression on histological specimens should be defined as HER2 negative cases, except in one case observed as "equivocal" in the histological specimen but as "amplified" in the CB by DISH assay.

There were two cases showing HER2 negative on CB but HER2 intermediate on the histological section. One of these cases should be categorized as a HER2 negative case because it was not amplified by HER2 DISH in both the histological specimen and the CB section. The cause of discrepancy of the other case was that the background signals seen on the histological section led to miscounting of the real HER2 signal. Therefore, these cases showing HER2 negative in CBs and HER2 intermediate in histological specimens should be categorized as HER2 negative cases.

Two cases were not used because of low numbers of cells on the CB slides. The reason of eliminating these cases is to use good quality samples for the data evaluation. The cellularity of these CBs was around 100. The IHC staining of these slides was evaluable. Therefore, 100 cells on a slide are enough amount of cells to be evaluable.

In summary, IHC staining of HER2 from breast cancer biopsies can be performed in CBs in the same way as that in histological specimens, although the number of equivocal cases in CBs may be more than that in histological specimens.

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