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Title Page

Title: Immunocytochemical results for HER2 and Ki67 in breast cancer touch-smear cell specimens are reliable

Masami Morimoto[1]

Yoshimi Bando[2]

Misako Nakagawa[1]

Hirokazu Takechi[1]

Takahiro Yoshida[1]

Junko Honda[3]

Yukiko Tadokoro[1]

Takuya Moriya[4]

Mitsunori Sasa[5]

Akira Tangoku[1]

[1]Depertment of Thoracic, Endocrine surgery and Oncology, Institute of Health Bioscience, The University of Tokushima Graduate School, Tokushima, Japan

[2]Division of Pathology, Tokushima University Hospital, Tokushima, Japan

[3]Higashi Tokushima Medical Center, Tokushima, Japan

[4]Department of Pathology, Kawasaki Medical School, Okayama, Japan

[5]Tokushima Breast Care Clinic, Tokushima, Japan

Correspondence author: Masami Morimoto, M.D., D.V.M.

Mailing address: Department of Thoracic, Endocrine surgery and Oncology, Institute of Health Bioscience, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima City, Tokushima, 770-8503, Japan

Tel: +81-88-633-7143

Fax: +81-88-633-7144

E mail: twop8air@tokushima-u.ac.jp

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Abstract

Background: Re-evaluation of the subtype of recurrent breast cancer is necessary for deciding the treatment approach, but it is often not performed due to the difficulty of obtaining tissue specimens from a recurrent lesion, etc. However, when a recurrent lesion is close to the body surface, fine-needle aspiration cells (FNA cells) can be easily obtained, and immunocytochemical (ICC) analysis of hormone receptors expression in FNA cells is said to be highly reliable. However, there is no consensus regarding ICC analysis of human epidermal growth factor receptor type2 (HER2) expression and the Ki67 index using FNA cells.

Methods: Touch-smear cells (TSC) were prepared from resected specimens from 36 patients with primary invasive ductal carcinoma of the breast. The TSC were fixed in 95% ethanol and subjected to ICC analysis for HER2 using HercepTest^{TM} (Dako) and Ki67 using MIB-1^{TM} (Dako). HER2 expression and the Ki67 index for the TSC were compared with the results of immunohistochemical analysis of histological section (HS). Statistical analyses used the kappa test and Pearson's correlation coefficients.

Results: HER2 and Ki67 were analyzed in TSC from 36 and 28 patients, respectively. The HER2 expression scores in the TSC and HS groups showed good agreement (kappa value=0.640), and significant correlation (correlation coefficient= 0.860, $p<0.001$). The

Ki67 indexes in the TSC and HS groups also showed significant correlation (correlation coefficient = 0.861, $p<0.001$).

Conclusions: The reliability of ICC analysis of HER2 expression and the Ki67 index using TSC were recognized.

Introduction

Subtype classification of breast cancer based on gene expression is important for predicting the results of therapy. The treatment approach for neoadjuvant and adjuvant chemotherapy is decided by immunohistochemical (IHC) analysis of estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor type2 (HER2) and Ki67 in the primary lesion. However, there is a possibility that the cancer cell subtype in recurrent breast cancer patients has changed from the subtype at the time of the surgery. For that reason, in order to design a more effective treatment approach following recurrence, it is desirable to re-evaluate the subtype at a recurrent site. In fact, however, such re-evaluation is often not performed, because it is technically difficult to obtain an excision biopsy or needle biopsy, or because the procedure would be too invasive, etc. As a result, even today, therapy for recurrent breast cancer is often decided on the basis of the subtype of the primary lesion.

Fine-needle aspiration (FNA) is comparatively non-invasive, and it can be performed even in cases when core needle biopsy is difficult, as long as a recurrent lesion is located close to the body surface. Significant concordance was reported for hormone receptor expression results generated by immunocytochemical (ICC) analysis of FNA cells and by IHC analysis of histological section (HS) [1]. However, opinion remains

divided with regard to the reliability of analysis of HER2 expression [2]. Accordingly, as a preliminary step to studies using FNA cells, we prepared touch-smear cells (TSC) from resected specimens obtained during surgery since that technique is able to collect large numbers of cancer cells and also yield uniform cell smears. We then determined HER2 expression in the TSC by ICC analysis and in HS by IHC analysis and compared the results. Moreover, although the Ki67 index is also important for subtype classification, there have been very few studies of this by ICC analysis of FNA cells [3]. Thus, here, we also examined whether evaluation of the Ki67 index by ICC analysis of TSC can be substituted for evaluation of HS by IHC analysis.

Materials and Methods

This study was approved by the Institutional Review Board at The University of Tokushima Graduate School. Informed consent was obtained from all patients.

Thirty-six patients were diagnosed with primary, invasive ductal carcinoma of the breast between April through October of 2012 and underwent surgery at the Higashi-Tokushima Medical Center without having received any neoadjuvant chemotherapy. TSC were prepared from the tumor portions of specimens that were resected during the surgery, fixed in 95% ethanol for 16-20 hours, and examined for HER2 and Ki67 by ICC analysis. HER2 was analyzed using HercepTest^{TM} (Dako), while Ki67 was analyzed using MIB-1^{TM} (Dako) (dilution: 1:50; pretreatment: autoclaving). The staining method was the same as for ordinary IHC analysis of HS. In addition, we prepare the formalin-fixed, paraffin-embedded tissue specimens of the cut surface from which the TSC had been obtained, and IHC analyses of HER2 and Ki67 were performed using the same antibodies as used for the TSC. For both the TSC and HS, HER2 staining was judged according to the ASCO/CAP guidelines of 0 to 3+ [4]. The Ki67 index for TSC was determined by observing at least 100 tumor cells and calculating the percentage of positively stained cells. In the histological slides, percentage was evaluated counting 500-1000 positively stained invasive tumor cells.

The slides were evalulated and scored by one pathologist (Y.B.).

Statistics

The staining results generated by ICC analysis of TSC were compared with the results generated by IHC analysis of HS. The HER2 expression scores and the Ki67 indexes in the TSC and HS groups were cross-tabulated, and the kappa (κ) values were calculated between the two groups using the kappa test. Kappa values above 0.6 were considered to represent good agreement, between 0.4 and 0.6 were considered moderate and below 0.4 was considered fair, while below 0.2 reflected poor agreement. Pearson's correlation coefficients were calculated for the HER2 expression scores and the Ki67 indexes in the two groups. A p value of <0.05 was considered to represent a statistically significant difference.

Results

The clinical data and pathological features of the 36 patients were as follows. The age range was 32–77 y (mean: 58.8 y). The clinical stage was I in 21 cases, IIA in 9 cases and IIB in 6 cases. The cytology was suspicious for malignancy in 7 cases and malignant in the remaining 29 cases. The nuclear grade was 1 in 18, cases, 2 in 7 cases and 3 in 11 cases.

HER2 staining was performed on TSC from all 36 patients, whereas staining for Ki67 was performed for only 28 cases. In 8 cases, insufficient amounts of tumor cells were available for Ki-67 staining and priority was given to staining for HER2. The results show that HER2 staining scores of 0–1+, 2+ and 3+ were recorded for 23, 8 and 5 cases for which TSC were tested, and for 28, 4 and 4 cases for which HS were tested (Figure 1). For Ki67, a cut-off of 14% was used, and the TSC specimens showed 8 cases of less than 14% and 20 cases of 14% or more, while the HS specimens showed 7 cases of less than 14% and 21 cases of 14% or more (Figure 2). Table 1 shows the paired results comparing the HER2 expression scores in the TSC and HS specimens. The value of κ was 0.640, which represents good agreement. The Pearson's correlation coefficient was statistically significant (correlation coefficient = 0.860; $p<0.001$). However, 5 (17.9%) of the 28 cases with score 0-1 in the HS group were rated as score 2 in the TSC group.

Similarly, one of the 3 HS group cases with score 2 was rated as score 3 in the TSC group. Thus, the results showed that the HER2 staining intensity was 1 score higher in the TSC group than in the HS group for 6 (16.7%) of the 36 cases. Among the six cases, two cases showed the heterogeneity of HER2 expression in their invasive lesions and the other case had DCIS lesion with stronger expression than adjacent invasive carcinoma (Figure 3).

Figure 4 shows the regression curve for the Ki67 index in the two groups, and the correlation coefficient was 0.861 ($p<0.001$), indicating a statistically significant correlation.

Discussion

It is fairly common for the primary and recurrent lesions of breast cancer patients to show differences in their hormone receptor and HER2 expression statuses. Re-evaluation of the subtype of recurrent lesions can be helpful in deciding the treatment approach and is therefore recommended. However, this is not very often performed in general clinical practice, for various reasons: it can be technically difficult to sample tissues, such as when the recurrent site is an organ metastasis, etc., and patients may refuse invasive procedures [5]. On the other hand, fine-needle aspiration cytology places less physical burden on patients than needle biopsy, and if subtype classification can be achieved by using FNA cells then the therapeutic approach can be decided with little invasiveness to the patient.

We employed TSC to investigate ICC analysis of HER2 and Ki-67 expression as a preliminary step prior to using FNA cells. In the case of the touch-smear method, the same cells as in the portion of cancer tissue of HS can be obtained. The more uniform and the less overlapped cells might be observed in touch-smear than FNA smear.

The findings for expression of hormone receptors generated by ICC analysis of FNA cells is said to show significant concordance with the findings generated by IHC analysis of HS [1], although opinions vary with regard to detection of HER2 expression.

One possibility for differences of opinion regarding the reliability of the results of ICC analysis of HER2 is the conditions used to fix the samples. Table 2 summarized the eight reports of use of the HercepTest™ to perform immunocytochemical (ICC) analysis of FNA cells [6-13]. Four of those reports stated that HER2 staining in FNA cells was difficult [6, 7, 10, 11]. Hanley et al. and Williams et al. wrote that there were many false-positive results with ICC analysis of ethanol-fixed FNA cells, and that the HER2 expression results using FNA cells were not reliable [10, 11]. In addition, they found reliability lacking in the analysis using cell blocks prepared by fixing the FNA cells in 50% ethanol, followed by treatment with 10% formalin [10.11]. Several reports covered analyses that used an antibody other than HercepTest™ and reported more cases of false-positive results with ICC analysis of c-erbB-2 (HER2) in ethanol-fixed FNA cells compared with IHC analysis of formalin-fixed HS [14-16]. In contrast, Moriki et al. and Sumiyoshi et al. reported very high concordance for HER2 expression between HS and FNA cells even in the case of ethanol fixation [8, 9]. In 2012, Pegolo et al. also reported that ethanol fixation did not influence the results of ICC analysis of HER2 in FNA cells [13]. In particular, alteration of the antigenicity of histological specimens by formalin treatment can be imagined as one cause of the different findings. Kumar et al. reported obtaining 90% concordance even when they prepared cell block specimens that

had been treated with formalin following ethanol fixation (the concentration was not stated), as long as they used good quality control [12]. This warrants further investigation.

Next, let's consider the ease of evaluation of HER2 expression. The HER2 protein is expressed on the cell membrane. It is difficult to estimate the positively staining of the cell membrane when the cells are clustered and embedded in the plenty of blood. FNA cells are prone to be non-uniformity and overlapping. This can be thought of as one reason for the lack of reliability of HER2 evaluation using FNA cells.

The number of breast cancer cases analyzed in this study was small, but our results showed significant agreement for ICC analysis of HER2 between the TSC group and the HS group, as indicated by a κ value of 0.640 and a correlation coefficient of 0.860 ($p<0.001$). However, we also found that, for 16.7% of the tested cases, the HER2 staining intensity was 1 score higher in the TSC group than in the HS group. Therefore, even when performing fixation with 95% ethanol, we cannot rule out the possibility of false-positive results for ethanol-fixed cell preparations, as Hanley et al. pointed out [10]. In the case of analyzing HS, there is a possibility that false-positives could be prevented by using normal ductal epithelium as an internal negative control, but normal ductal cells may not be included in the slides of cell specimens. It seems that we need a

new approach to reducing false-positives when testing cell specimens. Moreover, the cause of discordance between cytology and histology might be the heterogeneity of the breast cancer. It has been shown that HER2 is expressed in DCIS more frequently than in invasive carcinoma [17]. In the present study, there was one case containing DCIS with stronger HER2 expression than the adjacent invasive lesion.

ThinPrep (Cytyc Corp., Boxborough, MA) is a cytology procedure for ICC analysis of cell specimens. This is a CytoLyt fixative that uses ethanol as a liquid cell fixation method. Three published reports used this method to perform ICC analysis of FNA cells for HER2 [6, 7, 13]. Bedard et al. compared the results for FNA cells with those for HS and reported excellent specificity, with a positive predictive value of 34.8% and a negative predictive value of 97.5% [6]. However, the sensitivity was low, and they reported that reliability was not demonstrated. Similarly, Beatty et al. reported that the agreement rate with HS was κ=0.3–0.667 and concluded that this was insufficiently reliable for clinical use [7]. On the other hand, Pegolo et al. reported in 2012 that found 100% agreement between their FNA cells results and HS results, and their method was very interesting [13].

The Ki67 index is also important for subtype classification of breast cancer. The only published paper regarding the Ki67 index determined by ICC analysis of cell specimens

is that of Rita et al., who reported 70% concordance [18]. Our results showed that the correlation coefficient for the Ki67 index between the TSC and HS groups was 0.861 ($p < 0.001$), i.e., strong correlation. Ki67 staining is a nuclear stain, and it can be surmised that sufficient reliability will also be obtained even in the case of ICC analysis of cell specimens.

In our present study, we investigated HER2 expression and the Ki67 index determined by ICC analysis of TSC as a preliminary step to studies using FNA cells. Our results showed that evaluation of HER2 expression and the Ki67 index in TSC can be reliably performed. With the aim of bringing this method to practicality in the clinic, it will now be necessary to perform ICC analysis using FNA cells and investigate whether the results agree with the results of IHC analysis using HS.

Conflict of Interest

All authors declare that they have no conflict of interest.

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Table 1. Paired results of HER2 score in touch-smear cells and histological sections

κ=0.640*		HER2 score in histological sections			
		0-1	2	3	Total
HER2 score in touch-smear cells	0-1	23	0	0	23
	2	5	3	0	8
	3	0	1	4	5
	Total	28	4	4	36

*Moderate pairwise κ agreement was demonstrated.

Table 2. Articles on the HER2 protein expression on FNAC specimens published after 2000

Author(year)	Number of cases	Target of comparison	Cytologic material	Fixative solution Antibody	Conclusion	Yes/No
Bedard YC,et al (2003) [5]	63	IHC	FNA cells	·CytoLyt fixative ·Hercep Test™(DAKO)	Low specificity	No
Beatty BG, et al (2004) [4]	51	IHC	FNA cells	·ethanol/formalin/CytoLyt fixative ·Hercep Test™(DAKO)	κ=0.3-0.667 Insufficiently reliable for clinical use	No
Moriki T, et al (2004) [5]	65(imprint) 45(FNAC)	IHC	imprint FNA cells	·absolute ethanol ·Hercep Test™(DAKO)	Concordance 100%	Yes
Sumiyoshi K, et al (2006) [6]	58	IHC	FNA cells	·95%ethanol ·Hercep Test™(DAKO)	Correlation coefficient=0.89(P<0.01)	Yes
Hanley KZ, et al (2009) [7]	41	IHC	FNA cells	·50%ethanol+10%formalin ·Hercep Test™(DAKO)	κ=0.45 Correlation coefficient=0.65 (P<0.0001)	No
Williams SL, et al (2009) [8]	34	IHC	FNA cells	·50%ethanol+10%formalin ·Hercep Test™(DAKO)	κ=0.571 Correlation coefficient=0.56 (P<0.002)	No
Kumar SK, et al (2011)[9]	50	IHC	FNA cells	·formalin ·Hercep Test™(DAKO)	Concordance 90%	Yes
Pegolo E, et al (2012)[10]	100	IHC	FNA cells	·CytoLyt fixative ·Hercep Test™(DAKO)	Concordance 100%	Yes

*IHC:immunohistochemistory; **FISH:fluorescence *in situ* hybridization; ***FNA cells: fine needle aspiration cells.

Figure Legends

Figure 1: HER2 status in touch-smear cells demonstrating 0 (a), 1+ (b), 2+ (c) and 3+ (d).

Figure 2: Ki-67 status in touch-smear cells demonstrating less than 14% (a) and 14% or more (b).

Figure 3: HER2 expression in the case demonstrating touch-smear specimen (2+) (a) and histological specimen with DCIS component (2+) surrounded invasive carcinoma (1+) (b).

Figure 4: The regression curve for the Ki67 index in the touch-smear-cell group and tissue-block group. The correlation coefficient was 0.861 ($p<0.001$), indicating a statistically significant correlation.

Figure. 1

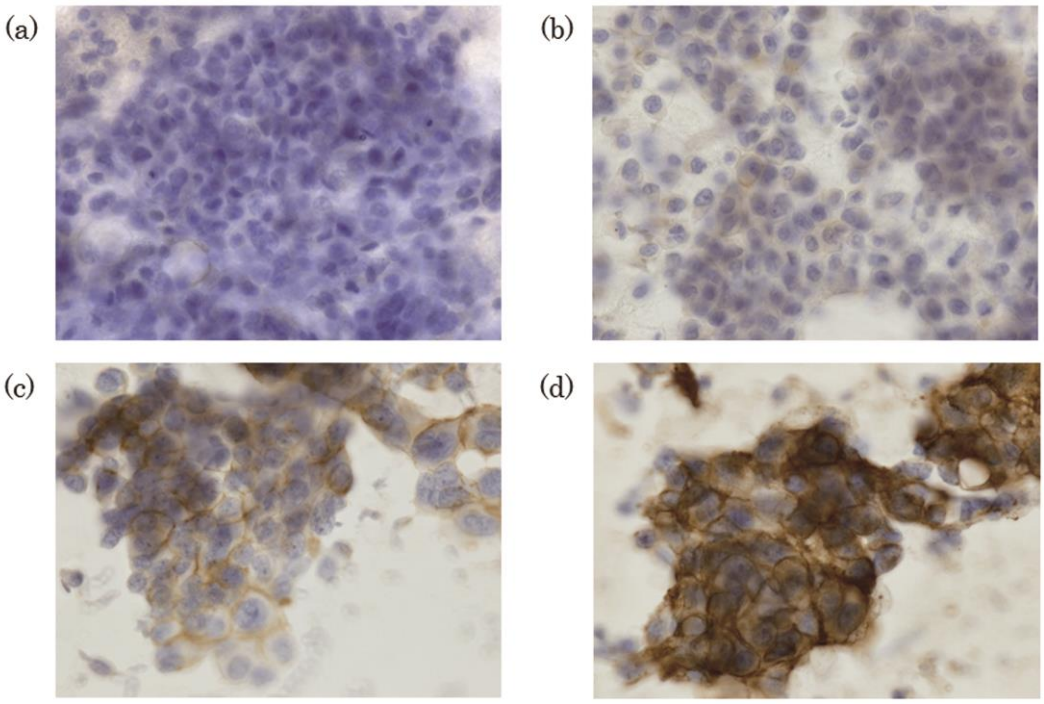

Figure. 2

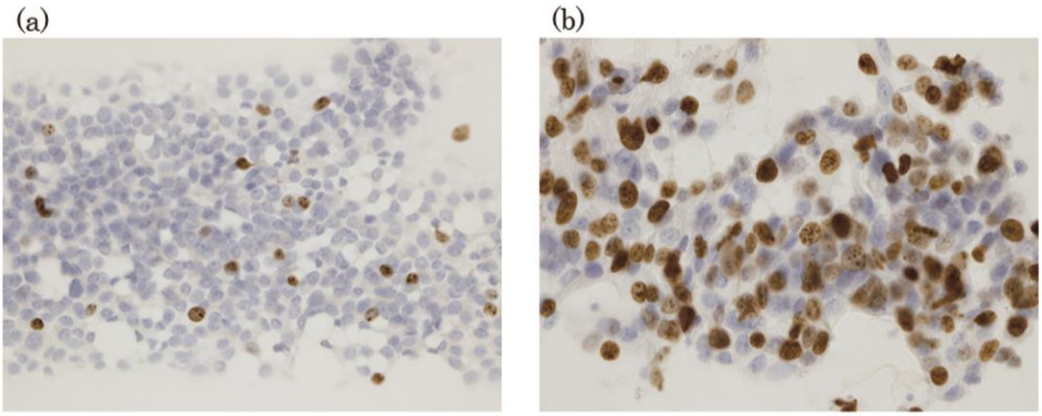

Figure. 3

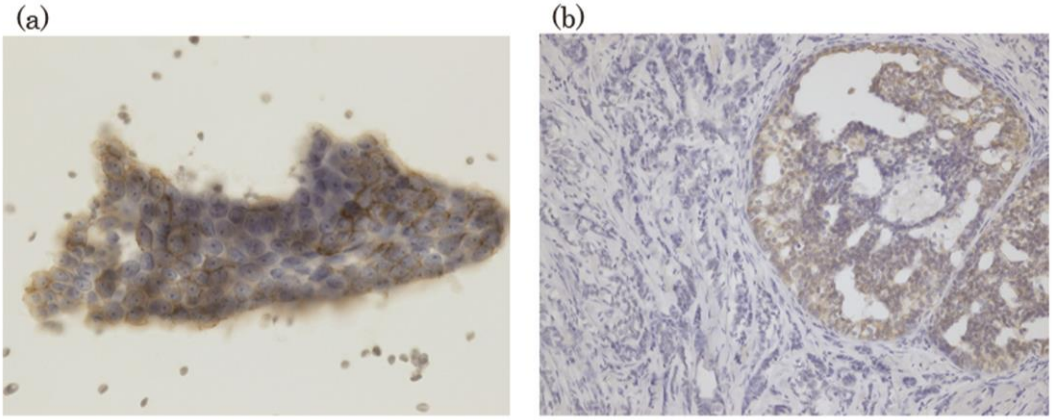

Figure. 4