

Detection of human epidermal growth factor receptor 2 protein and gene in fine needle aspiration cytology specimens and tissue sections from invasive breast cancer: Can cytology specimens take the place of tissue sections?

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Abstract. Overexpression of HER2 protein and *HER2* gene amplification in breast cancer are prognostic factors for the response to specific medical treatments such as trastuzumab, endocrine therapy, and chemotherapy. Whereas HER2 expression and gene amplification are generally examined in tissue sections, we investigated whether specimens from fine needle aspiration cytology (FNAC) are adequate for these analyses. HER2 protein overexpression and *HER2* gene amplification were assessed in both FNAC specimens and tissue sections from 58 cases of invasive breast cancer. Immunohistochemistry assay for HER2 protein expression was performed according to the HercepTest protocol, and *HER2* gene amplification was examined with the Spot-light CISH (chromogenic *in situ* hybridization) Detection kit. There was a significant positive correlation between assessments of HER2 protein status in the cytology specimens and tissue sections. The sensitivity, specificity, and accuracy of *HER2* gene amplification detection in cytology specimens in relation to those in tissue sections were 84.0% (21/25 cases), 87.9% (29/33 cases), and 86.2% (50/58 cases), respectively. FNAC specimens are suitable for detection of HER2 overexpression and *HER2* gene amplification in invasive breast cancer.

Introduction

Overexpression of HER2 protein and *HER2* gene amplification in breast cancer are prognostic factors for the response to

specific medical treatments such as trastuzumab, endocrine therapy, and chemotherapy (1-7). *HER2* gene amplification or HER2 protein overexpression has been identified in 20-30% of human breast cancers (8,9). HER2 expression and gene amplification are generally examined in tissue sections; however, it would be beneficial if cytology specimens could be used because the method used to obtain such specimens is minimally invasive. Even in tissue samples obtained by core needle biopsy, it is often too difficult to assess HER2 status because the samples are very small or the cancer cells are crushed. Although some studies of HER2 status in cytology specimens have been reported (10-14), assessment of HER2 status in the cytology specimen has not been clearly defined. Moreover, no studies comparing cytology specimens and tissue sections with respect to HER2 expression and *HER2* amplification have been reported. Therefore, the goal of the present study was to determine whether fine needle aspiration cytology (FNAC) specimens can be used for the assessment of *HER2* gene amplification and HER2 overexpression.

Materials and methods

Patients. The study population consisted of 58 patients who underwent surgery for primary invasive breast cancer at Osaka Medical College (Japan). Patient and tumor characteristics are shown in Table I. The patients comprised 1 man and 57 women who ranged in age from 32-84 years. The tumors included 20 papillotubular carcinomas, 6 solid tubular carcinomas, 31 scirrhous carcinomas, and 1 mucinous carcinoma. During surgery, we used a fine needle to obtain an aspirate from the central part of the resected tumor, and prepared cytology specimens fixed in 95% ethanol. We later prepared sections from formalin-fixed, paraffin-embedded tissue specimens from the same cases. We compared HER2 status in cytology specimens and tissue sections. Histologic type was determined from sections of the central part of the tissue specimens.

Immunohistochemistry (IHC). IHC was done with the HercepTest (DakoCytomation). Tissue sections and cytology

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Key words: invasive breast cancer, HER2, gene amplification, chromogenic *in situ* hybridization, fine needle aspiration cytology specimen, tissue section

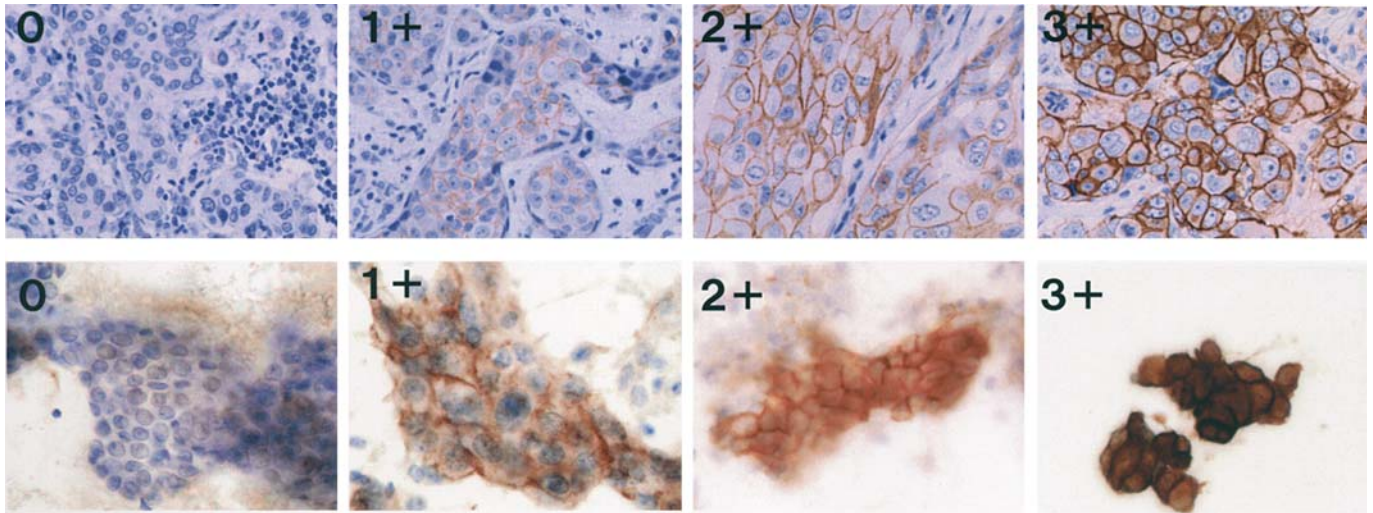


Figure 1. Criteria for scoring HER2 protein expression in tissue sections and FNAC specimens. Upper, tissue sections; lower, FNAC specimens. Samples were scored according to the HercepTest guidelines. Scores were: 0, no staining or staining of <10% of cancer cells; 1+, slight staining; 2+, intermediate staining; and 3+, strong staining of >10% of cancer cells.

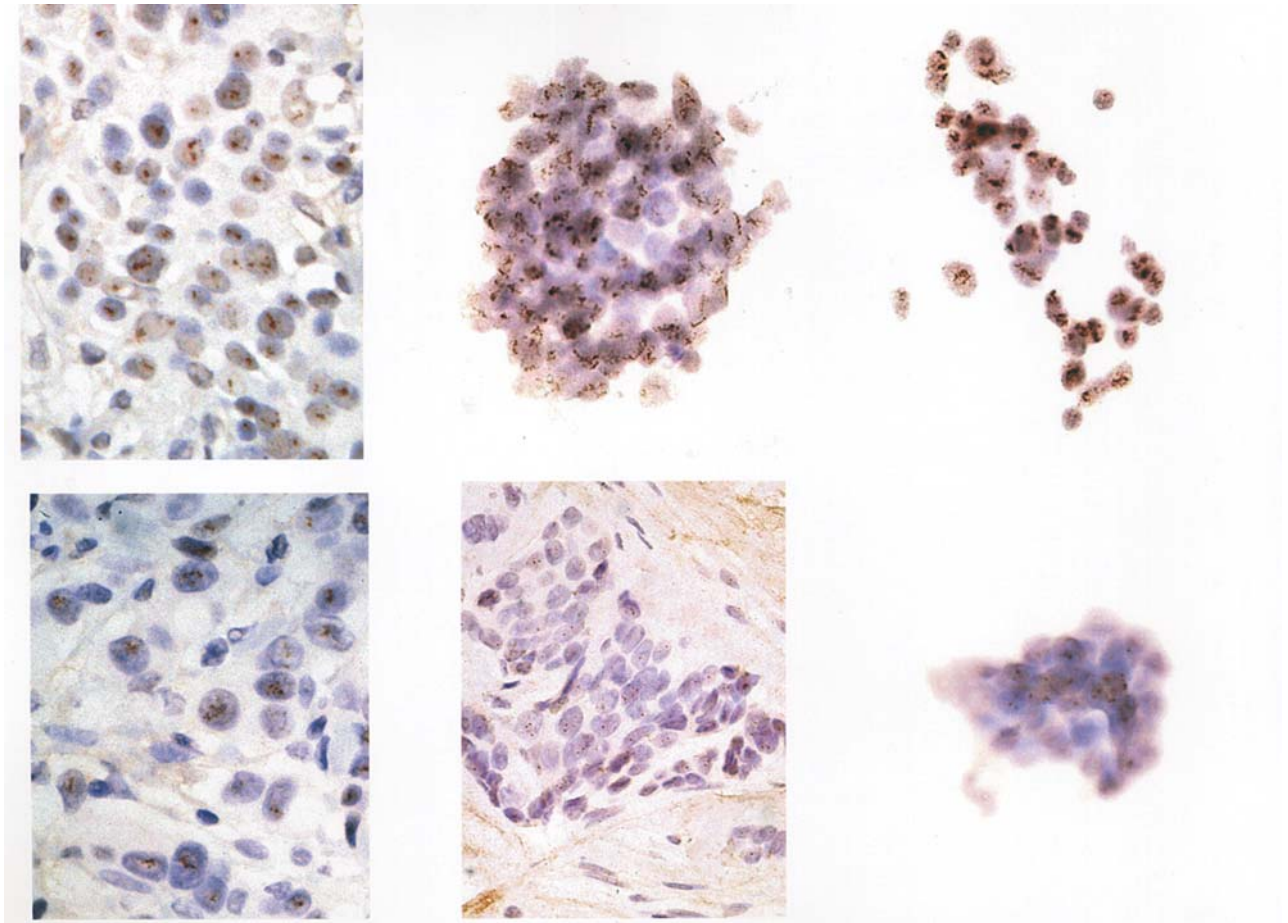


Figure 2. Criteria of *HER2* gene amplification (CISH). Upper left, typical *HER2/neu* amplification appearing as clusters (score A); tissue section. Upper middle, typical *HER2/neu* amplification appearing as multiple dots (score B); FNAC specimen. Upper right, typical *HER2/neu* amplification appearing as clusters and multiple dots (score C); FNAC specimen. Lower left, typical *HER2/neu* amplification appearing as 5 to 10 dots (score D); tissue section. Lower middle, no amplification. A tumor with 1 or 2 clearly identifiable copies of the *HER2/neu* gene; tissue section. Lower right, no amplification; FNAC specimen. Original magnification, x400.

specimens were reacted with the anti-HER2 polyclonal antibody provided with the HercepTest (A485), then visualized using the Envision method (DakoCytomation). Tissue sections were deparaffinized before antigen retrieval in

citrate buffer (pH 6.0) at 95°C for 40 min, then 20 min at room temperature. Antigen retrieval was not performed for the cytology specimens.

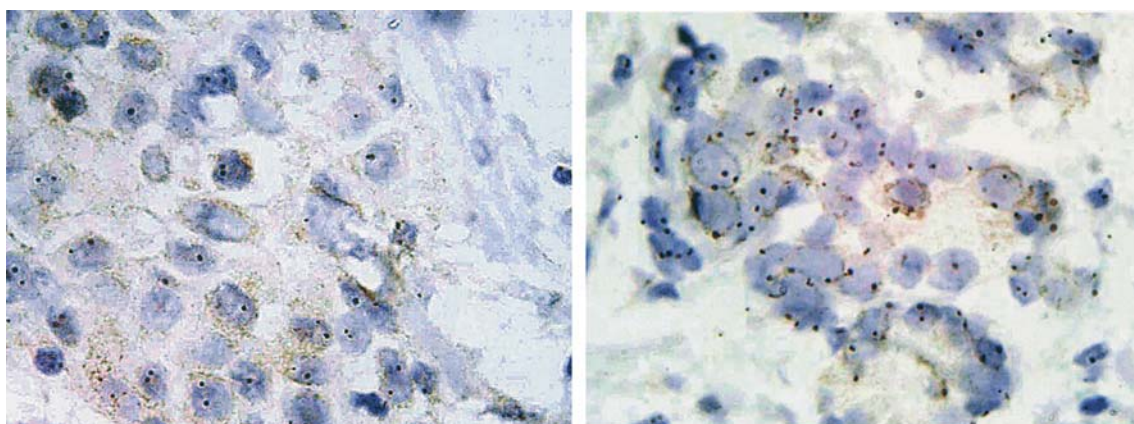


Figure 3. Chromosome 17 centromeric status. (Left) Two dots per cell: normal Chr 17; extra HER2 dots are caused by HER2 amplification. (Right) Three to 5 dots per cell: polysomy of Chr 17; extra HER2 dots are not caused by HER2 amplification.

Table I. Clinical characteristics of patients with invasive breast cancer.

Sex (M/F ratio)	1:57
Age (years)	32-84 (mean, 56)
Tumor size (mm)	12-140 (mean, 30)
Lymph node metastasis	
(-)	27
(+)	31
Invasive ductal carcinoma	
Papillotubular	20
Solid tubular	6
Scirrhou	31
Mucinous	1

Assessment of HER2 protein expression. HER2 protein levels were determined as described in the HercepTest manual (Fig. 1). Scores were: 0, no staining or staining of <10% of cancer cells; 1+, slight staining; 2+, intermediate staining; and 3+, strong staining; of >10% of cancer cells. Correlation between data from cytology specimens and tissue sections was evaluated with Spearman's rank correlation coefficient.

Chromogenic in situ hybridization (CISH). CISH of tissue sections was done according to the protocol of the Spot-light CISH Detection kit (Zymed Inc.). Sections were de-paraffinized, and incubated in a 95°C water bath for 15 min. Sections were then treated with proteinase K at room temperature for 10 min. Endogenous peroxidase activity was blocked with 3% H₂O₂ in methanol, and samples were then air dried. Digoxigenin-labeled HER2 probe (15 µl) was applied to the sections, which were then covered with coverslips. Slides were denatured on a hot plate at 80°C for 3 min, and hybridization was performed overnight (16-24 h) at 37°C. To remove the coverslips, the slides were soaked in 1X Tris-buffered saline containing 0.1% Tween-20. Slides were then washed with a stringent wash solution at 75°C for 5 min. Samples were blocked with normal serum, then incubated with mouse anti-digoxigenin and polymerized HRP-anti-mouse antibody for 60 min at room temperature. Binding was visualized with 3,3'-diamino benzidine.

For cytology specimens, a slightly modified protocol was used. Briefly, after enzymatic digestion, cells were re-fixed with 4% paraformaldehyde at room temperature for 15 min. Slides were denatured on a hot plate at 90°C for 3 min and hybridized overnight at 37°C. Slides were then washed with a stringent wash solution (1:10 dilution of standard sodium citrate solution) at 50-55°C for 5 min. All other procedures were as described for tissue sections. Positive controls were included in each staining run.

Table II. HER2 protein overexpression and HER2 gene amplification in tissue sections.

	IHC				Total
	0	1+	2+	3+	
CISH					
Amplified	2	1	6	16	25
Non-amplified	18	7	3	5	33
Total	20	8	9	21	58
Gene amplification rate (%)	10.0	12.5	66.6	76.2	43.1

IHC, immunohistochemistry.

Table III. HER2 protein overexpression and *HER2* gene amplification in FNAC specimens.

	IHC				Total
	0	1+	2+	3+	
CISH					
Amplified	0	1	10	14	25
Non-amplified	21	3	7	2	33
Total	21	4	17	16	58
Gene amplification rate (%)	0	25.0	58.8	87.5	43.1

IHC, immunohistochemistry.

Table IV. Comparison of FNAC specimens and tissue sections in IHC assay.

	IHC	Tissue sections			
		0/1+	2+	3+	Total
FNAC specimens	0/1+	25	0	0	25
	2+	3	6	8	17
	3+	0	3	13	16
	Total	28	9	21	58

FNAC, fine needle aspiration cytology; IHC, immunohistochemistry.

Table V. *HER2* gene amplification score in FNAC specimens and tissue sections.

FNAC specimens				Tissue sections			
Score	No. of cases	Chr17	No. of cases	Score	No. of cases	Chr17	No. of cases
A	0			A	2		
B	17			B	15		
C	4			C	5		
D	12	+	4	D	11	+	3
		-	8			-	8
No amplification		25				25	

FNAC, fine needle aspiration cytology; Chr17, chromosome 17.

Table VI. Comparison between FNAC specimens and tissue sections in the detection of *HER2* gene amplification.

		Tissue sections		Total (%)
		Positive (%)	Negative (%)	
FNAC specimens	Positive	21 (36.2)	4 (6.9)	25 (43.1)
	Negative	4 (6.9)	29 (50.0)	33 (56.9)
Total		25 (43.1)	33 (56.9)	58 (100.0)

FNAC, fine needle aspiration cytology.

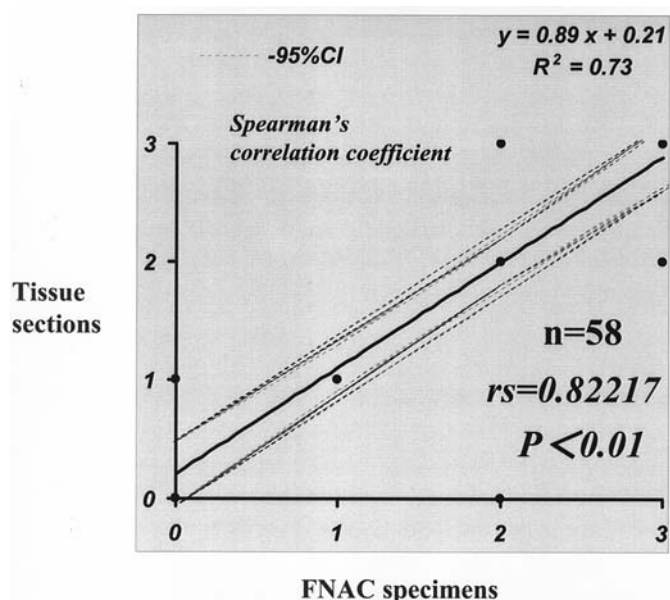


Figure 4. Correlation between HER2 protein expression on FNAC specimens and tissue sections. HER2 expression data from cytology specimens correlated significantly with those from tissue sections.

HER2 gene amplification. For both tissue sections and FNAC specimens, *HER2* amplification was assessed according to the Zymed *HER2* CISH Test Interpretation Guide, as shown in Fig. 2. Briefly, no amplification was defined as ≤ 5 *HER2* signals per nucleus in at least 50% of cancer cells. Amplification was defined as >10 dots or signals or large clusters of *HER2* per nucleus in at least 50% of the cancer cells. Scores were assigned as: A, clusters; B, multiple dots; and C, clusters and multiple dots. Low amplification (score D) was defined as 5 to 10 dots (or signals) of *HER2* per nucleus in at least 50% of cancer cells. In cases of low *HER2* amplification, we used biotin-labeled Spot-light Chromosome 17 Centromeric Probe (Chr17 cen probe) to confirm that the *HER2* signals of the gene were due to *HER2* gene amplification rather than polysomy of chromosome 17. When two signals were observed for Chr17 in a nucleus, we considered any extra *HER2* dots to be caused by *HER2* amplification (Fig. 3). We examined the correlations between the data from cytology specimens and tissue sections with a 2x2 table for sensitivity, specificity, and accuracy.

Results

HER2 expression. *HER2* protein scores for tissue sections are shown in Table II. Twenty cases had a score of 0, 8 cases had a score of 1+, 9 cases had a score of 2+, and 21 cases had a score of 3+. *HER2* protein scores for FNAC specimens are shown in Table III. Twenty-one cases had a score of 0, 4 cases had a score of 1+, 17 cases had a score of 2+, and 16 cases had a score 3+. Assuming that cases with a score of 1+ or 0 are in the same category, as the difference between score 0 and score 1+ is not clinically important, accuracy was 75.9% (44/58 cases) (Table IV). When histological type was considered, concordance between FNAC samples and tissue sections (assuming cases with a score of 1+ or 0 are in the

same category) was 75.0% (15/20 cases) for papillotubular carcinoma, 83.3% (5/6 cases) for solid tubular carcinoma, 77.4% (24/31 cases) for scirrhous carcinoma, and 0% (0/1 case) for mucinous carcinoma. There was a significant correlation between the cytology specimens and tissue sections with respect to accuracy in the analysis of *HER2* protein status [correlation coefficient, r_s , was 0.89 ($P < 0.01$, $n=58$)] (Fig. 4).

HER2 amplification. *HER2* amplification data for tissue sections are shown in Table V. Twenty-five cases (43.1%) showed gene amplification, and 33 cases showed no amplification. Among cases with amplification of *HER2*, 2 cases had a score of A, 15 cases had a score of B, 5 cases had a score of C, and 11 cases had a score of D. In 3 of the 11 cases with score D, low *HER2* amplification was confirmed with the Chr17 cen probe. Among tissue sections, the percentage of cases with *HER2* amplification relative to the protein score was 10.0% for score 0 (2/20 cases), 12.5% for score 1+ (1/8 cases), 66.7% for score 2+ (6/9 cases), and 76.2% for score 3+ (16/21 cases) (Table II). The *HER2* scores on FNAC specimens are shown in Table V. Twenty-five cases (43.1%) showed amplification of *HER2*, and 33 cases showed no amplification. Among cases with gene amplification, 0 cases had score A, 17 had score B, 4 had score C, and 12 had score D. Of the 12 cases with score D, 4 were confirmed to have low *HER2* amplification with the Chr17 cen probe. On FNAC specimens, the percentage of cases with amplification of *HER2* relative to the protein score was 0% (0/21 cases) for score 0, 25.0% (1/4 cases) for score 1+, 58.8% (10/17 cases) for score 2+, and 87.5% (14/16 cases) for score 3+ (Table III). With respect to histologic types, concordance was 85.0% (17/20 cases) in papillotubular carcinoma, 83.3% (5/6 cases) in solid tubular carcinoma, 87.1% (27/31 cases) in scirrhous carcinoma, and 100.0% (1/1 case) in mucinous carcinoma. The sensitivity, specificity and accuracy for detection of *HER2* gene amplification in cytology specimens in comparison to tissue sections were 84.0% (21/25 cases), 87.9% (29/33 cases), and 86.2% (50/58 cases), respectively (Table VI). Our results suggest that FNAC specimens can be used for detection of *HER2* amplification and overexpression in invasive breast cancer.

Discussion

Trastuzumab and trastuzumab with chemotherapy are more effective treatment strategies for cancers with *HER2* amplification than for those without amplification (1-4). For some studies, we found good concordance between CISH and FISH (15-20). The main difficulty in adopting FISH for clinical diagnostics is the need for fluorescence microscopy, which is not done in most routine diagnostic laboratories. Moreover, because fluorescence signals fade within a few weeks, hybridization results must be recorded with expensive digital cameras. To address these practical limitations, we used the CISH method because the probe can be detected with a simple IHC-like peroxidase reaction.

Although CISH-based analysis of *HER2* gene amplification has been reported for tissue sections of breast cancer (16,17,21), analysis of *HER2* amplification in cytology specimens has

not been reported. The aim of the present study was to determine whether FNAC specimens can be used to examine *HER2* gene amplification, and we thus compared the data from tissue sections and FNAC specimens.

We used the Spot-Light CISH Detection kit for analysis of cytology specimens. However, we were unable to obtain adequate signals and had to modify the manufacturer's protocol. These changes included a second fixation in 10% neutral buffered formalin for 1 min followed by 4% paraformaldehyde for 15 min, denaturation at 80°C for 3 min, then 90°C for 3 min, and a washing in a stringent solution sodium citrate at 72°C for 5 min followed by a washing in a 1:10 dilution of sodium citrate at 50–55°C for 5 min. With this modified protocol, we were able to obtain adequate signals on the cytology specimens.

The *HER2* gene amplification rate is high (43.2%) on FNAC specimens and tissue sections, as the relatively large size of tumors in the present study would suggest that they were examined for hormone receptor expression. Accordance between results in FNAC and tissue specimens did not differ among histologic types. With respect to *HER2* protein expression, all FNAC specimens with a score of 0 or 1+ (25 cases) had score of 0 or 1+ on tissue sections. Most (81.3%, 13/16 cases) FNAC specimens with protein scores of 3+ also had a score of 3+ on tissue sections, and the remaining 3 cases with a *HER2* protein score of 3+ showed amplification of the *HER2* gene on tissue sections. However, only 35.3% (6/17 cases) of FNAC specimens with a *HER2* protein score of 2+ had the same score on tissue sections. These findings suggest that for cases with *HER2* protein scores of 0, 1+, or 3+, FNAC specimens may be used instead of tissue sections without a loss of accuracy. However, the low accordance between FNAC specimens and tissue sections for cases with a score of 2+ indicates that FNAC cannot be substituted for tissue sections under these conditions.

With respect to *HER2* gene amplification, the sensitivity, specificity, and accuracy for the detection of *HER2* gene amplification in FNAC specimens compared with that in tissue sections were 84.0% (21/25 cases), 87.9% (29/33 cases), and 86.2% (50/58 cases), respectively. Four of the 25 cases with *HER2* gene amplification on tissue sections were negative for amplification on FNAC specimens. We believe that these false-negative results may have been due to sampling errors. In other words, the cells were not aspirated from the cancer nest. Increasing the quality of FNAC specimen by aspirating from several areas of the cancer may improve the accuracy of detection by FNAC. There were 4 of 33 cases that were negative for the *HER2* gene on tissue sections were positive for amplification on FNAC specimens. FNAC specimens from these 4 false-positive cases had an amplification score of D, and FNAC specimens had to be examined with a Chr17 cen probe. Of these 4 cases, 2 had a score of D on both FNAC specimens and tissue sections.

Because FNAC is a minimally invasive technique for obtaining tissue samples, we are able to easily obtain multiple specimens. With a combination of tests for some FNAC specimens, we can correctly assess *HER2* status during the pretreatment stage. Moreover, if the core needle biopsy specimen is small or if cancer cells are crushed, it is beneficial to assess the *HER2* status with FNAC specimens.

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