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Review

Evaluation of HER2 Status in Human Breast Carcinoma

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HER2 oncogene plays an important role in the oncogenesis and clinical behavior of 25–30% of human breast cancers. Recently, a recombinant humanized monoclonal antibody against HER2 protein, trastuzumab, has been administrated to patients with HER2 positive breast carcinoma. This antibody-based therapy is only effective in HER2-positive carcinoma cases, and therefore, it is very important to evaluate HER2 status with accuracy in order to determine the appropriateness of trastuzumab treatment for the breast cancer patients. Evaluation of HER2 status is mainly performed in the primary breast tumor tissues and several pathological methods, including immunohistochemistry, fluorescence *in situ* hybridization (FISH) and chromogenic *in situ* hybridization (CISH) which has been newly developed, are currently used in diagnostic laboratories. In this review, we describe the recent studies about these methods and discuss their reliability and possible further application to various pathological specimens obtained from breast carcinoma patients.

Key words: breast carcinoma, chromogenic *in situ* hybridization (CISH), fluorescence *in situ* hybridization (FISH), HER2, immunohistochemistry

I. Introduction

HER2 oncogene is located on the long arm of chromosome 17 at 17q21 and encodes a putative membrane tyrosine kinase receptor with homology with epidermal growth factor receptor. The HER2 gene is considered to be central to the oncogenesis and clinical behavior of 25–30% of human breast cancers [33, 34] and HER2 overexpression and/or gene amplification is a predictor for a worse clinical outcome for both node-positive [33, 34] and node-negative breast cancers [21]. Recently it has been demonstrated that a recombinant humanized monoclonal antibody against HER2 protein, trastuzumab (Herceptin^{R}; Genetich, South San Francisco, CA, USA) antagonizes the growth signaling properties of the HER2 system and/or enhances augmentation of chemotherapy in the breast carcinoma patients [30].

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As this antibody-based therapy is only effective in the carcinoma positive for HER2, it is very important to evaluate HER2 status with accuracy in order to determine which patients are candidates for trastuzumab treatment. Evaluation of HER2 status is mainly performed using archival pathological materials and several pathological methods, including immunohistochemistry, fluorescence *in situ* hybridization (FISH) and chromogenic *in situ* hybridization (CISH) which has been recently developed, are currently available in various laboratories. Therefore, in this review, we describe the recent evidence about these methods and discuss their reliability and possible further application to various pathological specimens in the patients with breast carcinoma.

II. Evaluation of HER2 Status in the Breast Carcinoma

Immunohistochemistry

Immunohistochemistry for HER2 protein is considered

to be the most attractive routine method based on the cost, convenience and biological relevance [6, 27, 30]. However, the immunohistochemical examination is possibly subject to technical variables, including the tissue fixation [20], antibody used and the evaluation system, which might affect the determination of trastuzumab therapy in the patients. Therefore, there is a need for standardization of HER2 assessment and reporting so that the results are comparable between laboratories [3].

Variability in sensitivity and specificity of commercially available HER2 antibodies used for immunohistochemistry has been previously noted [6, 22]. The different antibodies for HER2 yielded varying overexpression rates of HER2 from 17.2% to 42% in the breast carcinoma [11, 14, 19], which is considered to be one of the most important technical problems in the immunohistochemical analysis. In Japan, HercepTest (DAKO, Carpenteria, CA, USA), which is a ready-to-use kit including a polyclonal antibody and automated immunostaining system such as Benchmark (Ventana Medical systems, Tucson, AZ, USA) using CB11 monoclonal antibody (Ventana Medical systems, Tucson, AZ, USA) are approved by the Ministry of Health, Labor and Welfare for immunohistochemical evaluation of HER2 status in the breast carcinoma. In addition, there are various HER2 antibodies commercially available, including monoclonal (TAB250 (Zymed, South San Francisco, CA, USA) and SV2-61γ (Nichirei, Tokyo, Japan)) and polyclonal (A0485 (DAKO, Carpenteria, CA, USA) and Nichirei polyclonal (Nichirei, Tokyo, Japan)) antibodies. These antibodies are used for immunohistochemistry in the various diagnostic laboratories. Press *et al.* [22] have compared the sensitivities and specificities of 28 antibodies for HER2, and reported marked variation in sensitivities ranging from 6–82% and specificities from 92–100%. In the HER2 evaluation by HercepTest, high false-positive rates and a relative low interobserver agreement level have been noted [9, 26], although several groups believe that it is too low to cause concern [5, 39]. Thomson *et al.* [36] reported that TAB250 is the most sensitive antibody compared to CB11, HercepTest and A0485, however, low immuno-positivity by TAB250 (nearly half compared to other antibodies) has been also pointed out by several groups [29, 40]. The different epitopes which antibodies recognize and/or conditions of antigen-retrieval according to HER2 antibodies may affect the variability in immunoreactivity of HER2.

It is very important to standardize the evaluation of HER2 immunoreactivity to decrease the interobserver variation. To date, the grading system, which was proposed in the HercepTest, is the most widely used for immunohistochemical HER2 evaluation, which is based on the HER2 immunointensity. Briefly, HER2 immunoreactivity is classified into the following 4 groups in this system: 0, no immunostaining; 1+, faint staining in more than 10% carcinoma cells; 2+, moderately and/or incompletely circumscribed membrane-staining in more than 10% carcinoma cells, and 3+, strong and completely circumscribed membrane-staining in more than 10% carcinoma cells. Use of positive control samples [25, 36] or standardized figures such as "HER2 Atlas" by the "Pathology committee for optimal use of Trastuzumab" (http://www.her2.org/) [44] has the potential to improve the reliability of immunohistochemical evaluation for HER2 regardless of the antibody used. Some pathologists do not like the evaluation based on the immunointensity because it is more subjective and possibly affected by technical variables. Tsuda *et al.* [40] have evaluated immunohistochemical HER2 status according to the immunostaining area regardless of the intensity and showed that this evaluation system does not always reflect the gene amplification of HER2 in the carcinoma cells and is less preferable.

Table 1. *Comparison of immunohistochemical HER2 evaluation between two diagnostic laboratories in 50 breast carcinomas*

CB11 (Benchmark)		Laboratory A			
		0	1+	2+	3+
Laboratory B	0	16	4	1	0
	1+	7	10	3	0
	2+	0	0	2	2
	3+	0	0	0	5

$p<0.0001$.
Data represent the number of cases.
Immunohistochemistry for HER2 was independently performed by Benchmark automated immunostaining system with the CB11 antibody in two laboratories and evaluated by HercepTest system.

It has been reported that interobserver agreement is relatively low in the group of 1+ or 2+ compared to the group of 0 or 3+, in the evaluation by HercepTest system [36, 40]. We also detected a similar tendency ($p<0.0001$) when we compared the HER2 status in 50 breast carcinoma tissues two different laboratories (Table 1). At present, Trastuzumab therapy is considered in the breast carcinoma patients showing 3+ HER2 immunostaining but not in the patients showed 0 or 1+. However, it remains controversial whether a score of 2+ should be assessed as overexpression of HER2 as well as 3+, and the patients showing 2+ are often offered trastuzumab therapy. As the evaluation of 2+ is not considered to be very reproducible in the HercepTest system, further examinations are necessary to determine the trastuzumab treatment in these cases, including evaluation of HER2 immunoreactivity by multiple observers and/or other additional examinations such as FISH.

FISH

Overexpression of HER2 protein generally (more than 95%) results from the HER2 gene amplification [18, 34]. FISH is thought to be the most sensitive technique for the quantitative evaluation of HER2 gene status in the carcinoma cells. Two different FISH assays, INFORM™ (Ventana Medical systems, Inc, Tucson, AZ, USA) and PathVysion™ (Vysis, Inc, Downers Grove, IL, USA) are commercially available and they are approved by the United States Food

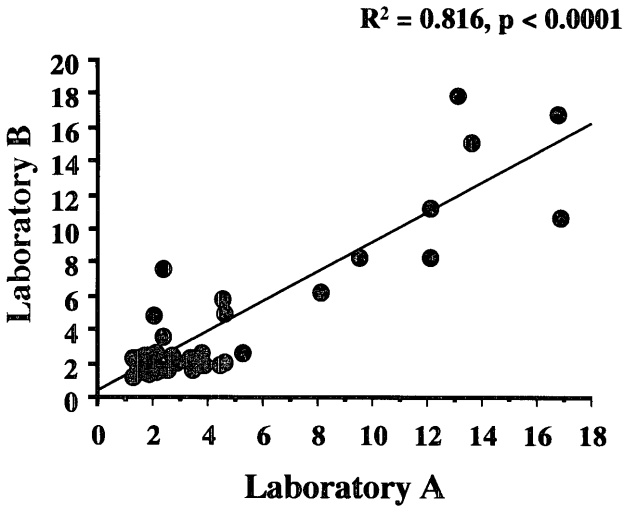

Fig. 1. Comparison of HER2 status by FISH (INFORM system) between two laboratories in 50 breast carcinomas. The FISH analysis was independently performed and evaluated in the two laboratories (Laboratory A and B). Data represent mean HER2 gene copy number per carcinoma nucleus in each case. Significant correlation (R^2=0.816, p<0.0001) was detected.

and Drug Administration (FDA). INFORM defines HER2 gene amplification as a mean HER2 gene copy number of more than 4.0 per carcinoma nucleus. PathVysion uses a double-probe system for simultaneous enumeration of HER2 genes and chromosome 17 centromers and HER2 gene amplification is evaluated as the ratio of HER2 gene copies per chromosome 17 centromer, *i.e.* more than 2.0 per carcinoma nucleus.

FISH quantifies the number of gene copies in the cancer cells and objectively reflects the HER2 gene status of the tumors, whereas immunohistochemistry is a relatively subjective test. Tsuda *et al.* [38] examined interobserver reproducibility of HER2 evaluation by FISH and reported that agreement level of HER2 amplification was nearly perfect between two observers regardless of the FISH systems. Similar results were also detected (R^2=0.816, p<0.0001) when we compared evaluation of HER2 status by the INFORM system between two laboratories (Fig. 1), demonstrating the potent interlaboratory reproducibility in the FISH analysis.

The use of probe for chromosome 17 centromer in the PathVysion system is meant to correct for HER2 pseudoamplification due to chromosome 17 polysomy [17, 23]. However, its clinical importance remains controversial. Pauletti *et al.* [19] examined 900 breast cancer patients using PathVysion FISH system and reported that chromosome 17 correction is essential for demonstration of HER2 gene amplification. McCormick *et al.* [15] found that 8.6% of breast tumors showed INFORM-positive but PathVysion-negative in 198 breast carcinomas. Conversely, Jimenez *et al.* [10] reported that discordance between HER2 and chromosome 17 copy number was not a useful means of defining amplification. Tubbs *et al.* [43] also showed that

Table 2. *Comparison of HER2 status between INFORM and PathVysion systems in 50 breast carcinomas*

		INFORM	
		No amplification	Amplification
PathVysion	No amplification	36	2
	Amplification	2	10

p<0.0001.
Data represent the number of cases.

the chromosome 17 correction rarely embellished or clarified HER2 FISH results in the examination of 400 breast carcinomas. When we compared HER2 status between INFORM and PathVysion systems in 50 breast carcinomas, significant correlation (p<0.0001) was detected between these two systems (Table 2).

Various studies have demonstrated that HER2 evaluation by FISH is strongly correlated with that by immunohistochemistry in the intensely positive (immunohistochemical score; 3+) or negative (immunohistochemical score; 0 or 1+) group, but not in the 2+ group. Therefore, the 2+ group is appeared to include both HER2-amplified and HER2-nonamplified tumors. Pauletti *et al.* [19] demonstrated that the survival probability of FISH-negative/immunohistochemistry-positive breast carcinomas was similar to that of FISH-negative/immunohistochemistry-negative cases and FISH-positive/immunohistochemistry-negative patients have a survival probability similar to that of FISH-positive/immunohistochemistry-positive cases. These data may partly explain the previous findings that patients with immunohistochemical score 2+ did not seem to benefit significantly from trastuzumab therapy [19]. Therefore, FISH is considered especially useful to examine the detailed HER2 status in the group of immunohistochemical score 2+.

CISH

FISH is one of the most reliable methods to evaluate the HER2 status at the DNA level, as described above. However, it is also true that FISH is a relatively cumbersome procedure and it requires technical skill and high cost. Therefore, it is not easily accommodated in the usual diagnostic surgical pathology practice [42]. CISH is a recently developed method, in which a chromosomal DNA probe is detected using an immunohistochemical-like peroxidase reaction rather than fluorescence as in FISH [12, 35]. Although CISH is considered to have a slightly lower sensitivity than FISH, it has the following advantages similar to the immunohistochemistry [35]: (1) fluorescence microscopy is not required, (2) signal intensity does not diminish over time, (3) it is easy to correlate the findings with the histology, (4) the method is easier than FISH and (5) it is more economical than FISH.

In CISH analysis, HER2 status is evaluated by the criteria of Tanner *et al.* [35]. Briefly, no amplification, less than

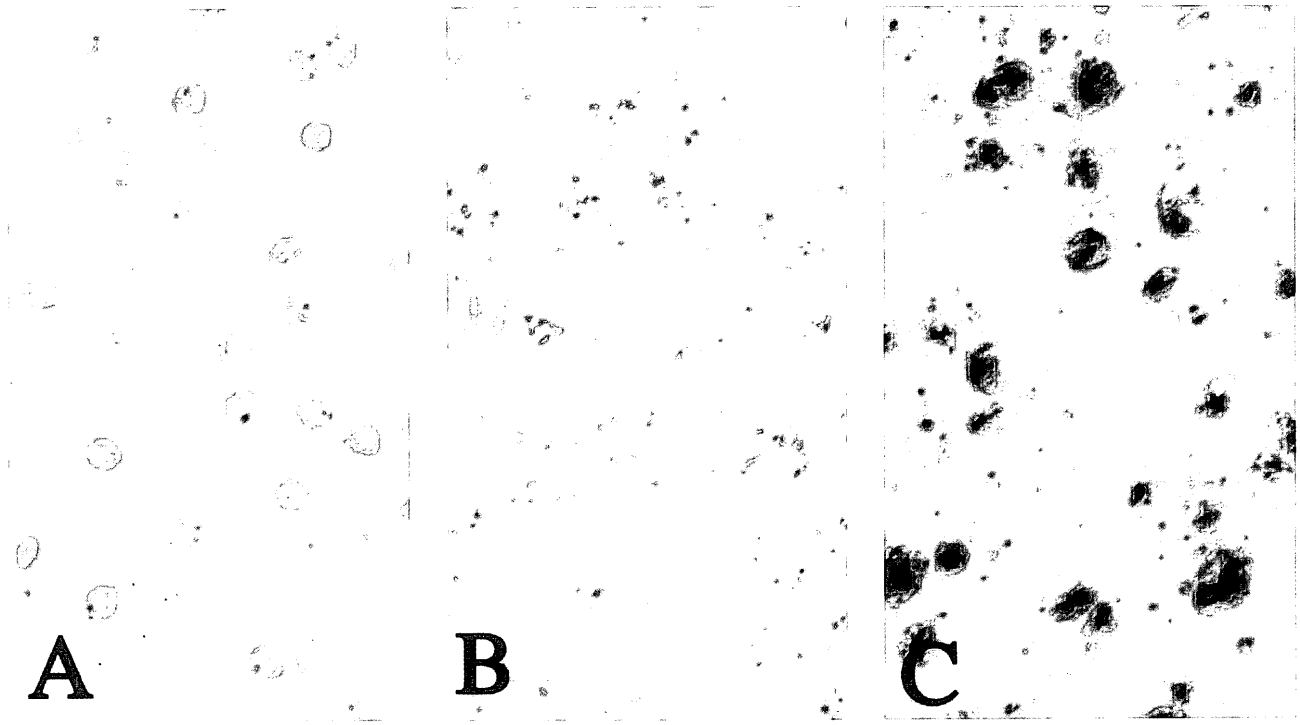

Fig. 2. HER2 evaluation by CISH based on Tanner *et al.* (2000) in the breast carcinoma. **A:** no amplification, less than 6 gene copies per nucleus, **B:** low-amplification, 6–10 gene copies per nucleus or small clusters in more than 50% carcinoma cells and **C:** high amplification, more than 10 gene copies per nucleus or large clusters in more than 50% carcinoma cells. INFORM HER2 probe was used and it was visualized by 3.3'-diaminobenzidine (DAB) solution. Counterstaining was performed by hematoxylin. ×560, respectively.

6 gene copies per nucleus; low-amplification, 6–10 gene copies per nucleus or small clusters in more than 50% of carcinoma cells; and high amplification, more than 10 gene copies per nucleus or large clusters in more than 50% of carcinoma cells (Fig. 2A–C). It has been reported that HER2 evaluation by CISH is well correlated with that by immunohistochemistry [12] (n=20) or FISH [45] (n=62). Gene amplification of HER2 was evaluated in more than 50% of carcinoma cells in CISH analysis, which is the same as United States FDA-approved FISH tests. Considering that evaluation of HER2 immunoreactivity is performed in more than 10% of positive cells, evaluation of HER2 status by CISH may be carried out for smaller number of tumor cells.

It has been reported that the signal intensity in CISH is influenced by the quality of tissue samples and/or pretreatment of sections [12, 35] and positive controls are very important to improve the reliability of CISH analysis. In addition, simultaneous enumeration of HER2 genes and chromosome 17 centromers is difficult in the same slide in CISH rather than FISH. CISH has a newly developed method and there are no large comparisons between CISH and immunohistochemistry or FISH yet. Further examinations are required to apply CISH to the routine diagnostics.

III. Application of HER2 Evaluation inVarious Pathological Specimens

Metastatic and recurrent tissues

As trastuzumab is an active agent for the treatment of metastatic breast cancer, HER2 status of carcinoma cells at the time of recurrence may greatly influence the response of trastuzumab treatment. However, sufficient amounts of specimens in these patients are not necessarily available for examination at the laboratories and the assessment of HER2 status for obtaining the information for the treatment decision is usually performed in the specimens obtained at the time of initial surgery [24]. Therefore, it is very important to know whether HER2 status in the recurrent lesions is the same as that in the primary breast carcinoma.

The alternation of HER2 expression is thought to occur in the early stage of the progression of breast cancer and to be maintained throughout cancer progression [8, 13]. Inglehart *et al.* [7] showed a good correlation of HER2 gene status between the primary and regional lymph nodes in 23 cases and Simon *et al.* [32] also reported that 77% of HER2-positive primary tumors had entirely HER2-positive metastases. When we compared the HER2 status between primary breast carcinoma tissues and synchronous metastatic lesions of lymph nodes in 17 cases, significant correlation was detected both in immunohistochemistry ($p=0.0002$) (Table 3) and FISH ($R^2=0.919$ and $p<0.0001$).

Table 3. *Comparison of immunohistochemical HER2 status between primary and synchronous metastatic lesions of lymph nodes in 17 breast carcinoma patients*

	CB11 (Benchmark)	Primary tumor			
		0	1+	2+	3+
Lymph node metastasis	0	6	2	0	0
	1+	0	2	1	0
	2+	0	0	1	0
	3+	0	0	0	5

p=0.0002.
Data represent the number of cases.
Immunohistochemistry for HER2 was performed by Benchmark automated immunostaining system with the CB11 antibody.

Table 4. *Comparison of immunohistochemical HER2 status between primary and asynchronous recurrent lesions in 19 breast carcinoma patients*

	CB11 (Benchmark)	Primary tumor			
		0	1+	2+	3+
Recurrent lesions	0	12	0	0	0
	1+	1	1	0	0
	2+	0	0	0	2
	3+	0	0	1	2

p=0.0003.
Data represent the number of cases.
Immunohistochemistry for HER2 was performed by Benchmark automated immunostaining system with the CB11 antibody.
Follow up times: 1–228 months.

These results are in agreement with previous reports described above and it is suggested that HER2 status of primary breast tumors is maintained in the majority of the metastases.

Only a few studies have been reported regarding comparisons between the primary breast tumor and asynchronous recurrent lesions in the same patients. Shimizu *et al.* [31] reported no discord in HER2 expression between the primary and matching recurrent lesions in 21 breast cancers. Similar findings were also shown by Tsutsui *et al.* [41]. When we examined the correlation of HER2 status between primary and asynchronous recurrent lesions by immunohistochemistry in 19 patients, significant correlation was detected (p=0.0003) regardless of the period of disease-free survival (Table 4).

Cytological specimens

Various cytological specimens are frequently available for examination of primary breast tumors or the recurrent sites and therefore, it is very important to apply the HER2 evaluation to these cytological materials. Fine needle aspiration (FNA) cytology is well known to be useful to evaluate the clinically detected suspicious lesions of the breast. Several groups have reported the immunodetection of HER2 on FNA smears. Troncone *et al.* [37] have found a higher incidence of HER2 overexpression in FNA smears than in surgical tissue specimens of breast cancer and discussed that it is possibly due to the better antigen preservation in the fresh cytological preparations. Corkill and Katz [4] also reported similar results. FISH analysis can be performed on cytological material from FNA [2, 16, 28] and Mezzelani *et al.* [16] showed a good correlation of HER2 status between FISH and immunohistochemistry.

Table 5. *Comparison of immunohistochemical HER2 status between primary lesions and recurrent carcinoma cells obtained from effusions (cytological specimens) in 11 breast carcinoma patients*

	CB11 (Benchmark)	Primary tumor			
		0	1+	2+	3+
Recurrent lesions	0	6	0	0	0
	1+	0	1	0	0
	2+	0	0	0	1
	3+	0	0	1	2

p=0.0003.
Data represent the number of cases.
Carcinoma cells obtained from pleural effusion or ascites at the time of recurrence were fixed with 10% formalin buffer and embedded in paraffin-wax (cell block). Immunohistochemistry for HER2 was performed by Benchmark automated immunostaining system with the CB11 antibody.
Follow up times: 9–190 months.

Serous effusions are important cytological materials to evaluate the recurrence of breast cancer. Ascoli *et al.* [1] examined HER2 immunostaining in serous effusions and reported HER2 immunoreactivity was easily detected both in alcohol-fixed smears and cell blocks from formalin-fixed specimens. To obtain a better understanding of the significance of HER2 evaluation in cytological specimens, we compared the HER2 status between carcinoma cells obtained from pleural effusion or ascites at the time of recurrence and corresponding primary lesions in 11 patients with breast carcinoma. HER2 status of recurrent carcinoma cells in cytological specimens was significantly correlated with that in primary tumor both in immunohistochemical (p=0.0003) (Table 5) and FISH analyses. These findings are consistent with previous studies using recurrent surgical tissues described in the above section [31, 41]. Therefore, it is suggested that cytological specimens are available for HER2 evaluation both by immunohistochemistry and FISH, which may provide further information about the characteristics of tumors or determination of trastuzumab treatment.

IV. Conclusions

It is very important to accurately evaluate the HER2 status in the breast carcinoma tissue to determine the trastuzumab therapy. HER2 status is mainly evaluated by pathological examinations, including immunohistochemistry and FISH. CISH is a newly developed method which may also become a useful tool for the HER2 evaluation in the near

future. Results from these analyses for HER2 are generally reliable and reproducible and are markedly correlated in the group of intensely positive or negative. However, some discrepant results were detected in the group of low overexpression of HER2, suggesting that detailed examinations are required to determine the trastuzumab therapy in these cases. As trastuzumab is used for the treatment of metastatic breast carcinoma patients, HER2 status of carcinoma cells at the time of recurrence may be very important to predict the response to the trastuzumab treatment. HER2 status in metastatic or recurrent lesions is closely correlated with that in the primary site, suggesting maintenance of HER2 status throughout the cancer progression. Cytological specimens are also available for examination of HER2 status, and application of HER2 evaluation to cytological specimens may become useful method to provide further information about the characteristics of breast tumors or the determination of trastuzumab treatment.

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