Stability of HER2 Status by Dual-color in Situ Hybridization Before and After Neoadjuvant Chemotherapy in Breast Cancer

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Objective: Trastuzumab may convert human epidermal growth factor receptor-2 (HER2)-positive primary breast tumors to HER2-negative tumors after chemotherapy. This study determined whether trastuzumab increases the number of patients with conversion to HER2-negative status and assessed the effect of neoadjuvant chemotherapy.

Methods: We retrospectively reviewed 46 patients diagnosed with HER2-overexpression in primary breast cancers at Tokai University Hospital, receiving neoadjuvant chemotherapy by immunohistochemistry and fluorescence in situ hybridization (FISH). Surgical specimens of patients achieving less than pathological complete response (pCR) were verified for sufficient residual tissue to evaluate post-treatment HER2 status by dual-color in situ hybridization (DISH).

Results: pCR was achieved in 8 of the 46 (17.4%) patients. The residual tumor was sufficient for assessing post-treatment HER2 status in 25 patients. DISH of pretreatment specimens confirmed HER2 amplification prior to therapy. Of the 25 HER2-positive patients, DISH revealed 3 were HER2 negative in pretreatment specimens. No post-treatment tumors were HER2-negative according to DISH in HER2-positive pre-treatment tumors. Among HER2-negative pretreatment tumors, 1 post-treatment tumor was HER2 positive and 2 had stable HER2 status.

Conclusion: HER2 status determined by DISH was stable between pretreatment breast tumors and residual tumors. However, a small discrepancy regarding HER2 status determined by immunohistochemistry and DISH existed.

Key words: In situ hybridization, Neoadjuvant therapy, Breast cancer, Chemotherapy, HER2

INTRODUCTION

The human epidermal growth factor receptor-2 (*HER2/neu* or *HER2*) gene is amplified in 20–25% of primary breast cancer cases. HER2-targeted therapies, such as trastuzumab as neoadjuvant chemotherapy for HER2-positive patients, result in better pathological complete response (pCR) than HER2-non-targeted therapy [1, 2]. Patients who do not achieve a pCR after neoadjuvant chemotherapy have poor prognosis than patients who receive therapy [3–7].

HER2 status may be discordant in primary breast tumors before and after chemotherapy [7–25]. Furthermore, some studies suggest that trastuzumab can convert disease status from HER2 positive in a primary tumor to HER2 negative in residual tumors [7, 20, 26–29]. However, Pusztai *et al*. suggest discordant results can be caused by limited accuracy and reproducibility of receptor assays [30].

HER2 status is compared by immunohistochemistry [7]. Mittendorf *et al*. reported that approximately one-third of patients with sufficient residual disease with repeated HER2 testing had lost *HER2* gene amplification according to fluorescence in-situ hybridization (FISH) analysis. Furthermore, patients who have lost *HER2* gene amplification had significantly lower relapse-free survival than patients whose tumors retained *HER2* gene amplification [29]. Patients with such HER2 discordance may have shorter survival duration than those with concordance between primary and residual or metastatic tumor sites [7, 29, 31].

Dual-color in-situ hybridization (DISH) is a novel in situ hybridization-based genetic test. *HER2* gene testing by DISH enables the observation of signals from both HER2 and chromosome 17 centromere (CEP17) on a single slide by using light microscopy [32]. The concordance between DISH and FISH for *HER2* gene diagnosis among 134 invasive breast cancer patients was 94.1%, 98.4%, and 96.2% for gene amplification patients, gene non-amplification patients, and all patients, respectively [33].

The extent of HER2 discordance between pre- and post-neoadjuvant chemotherapy in primary breast tumor has not been conclusively established, and it is unclear if trastuzumab increases this discordance.

Accordingly, we hypothesized that treatment with chemotherapy or with the HER2-targeted drug, trastuzumab increases the likelihood that patients with HER2-positive primary tumors have HER2-negative residual tumors. Therefore, this single-center study compared HER2 status determined by DISH in primary tumors of both pre- and post-neoadjuvant chemo-

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therapy in 25 patients with breast cancer.

MATERIALS AND METHODS

Patients

We retrospectively identified 46 patients from the Breast Cancer Database of Tokai University Hospital who had been diagnosed with primary HER2-positive (immunohistochemistry [IHC] 3+ and/or FISH positive) breast cancer and treated with neoadjuvant chemotherapy with or without trastuzumab between January 1, 2000, and December 31, 2010. Surgical specimens from patients achieving less than pCR were assessed to determine if there was sufficient residual tissue to evaluate the post-treatment HER2 status by DISH. This study was approved by the Institutional Review Board of Tokai University, which waived the need for written informed consent because of the retrospective nature of the study.

Neoadjuvant chemotherapy

Table 1 shows the neoadjuvant chemotherapy regimens. Twelve patients were administered docetaxel 75 mg/m^2 i.v. every 3 weeks followed by 4 cycles of epirubicin 90 mg/m^2 and cyclophosphamide 600 mg/m^2 i.v. administered on the first day of each cycle every 3 weeks. Nine patients were administered paclitaxel 80 mg/m^2 i.v. weekly for 12 weeks followed by 4 cycles of epirubicin 90 mg/m^2 and cyclophosphamide 600 mg/m^2 i.v. administered the first day of each cycle every 3 weeks. Two patients were administered paclitaxel 80 mg/m^2 i.v. weekly for 12 weeks followed by 4 cycles of adriamycin 90 mg/m^2 and cyclophosphamide 600 mg/m^2 i.v. administered the first day of each cycle every 3 weeks. Two patients were administered paclitaxel 80 mg/m^2 i.v. weekly for 12 weeks. Five patients were administered trastuzumab 8 mg/kg i.v. as a loading dose on the first day and 6 mg/kg every 3 weeks concomitant with taxane chemotherapy.

HER2 status measured by DISH

HER2 status was defined as positive if DISH demonstrated a gene copy ratio of *HER2/CEP17* ≥ 2.0. Paraffin tissue sections (4 μm thick) were mounted on glass slides (New Sliane III, Catalog No. 5126-25; Muto Pure Chemicals, Co. Ltd., Tokyo, Japan) and stained using the newly developed fully automated HER2 Dual ISH assay on a BenchMark® XT slide stainer according to the manufacturer's recommendations (Ventana Medical Systems Inc., Tucson, AZ, USA). The stained slides were subsequently rinsed with tap water containing neutral detergent and rinsed again with distilled water. The slides were dried at room temperature for at least 60 min and covered by cover glass for SGC (Muto Pure Chemicals). HER2 DISH and hematoxylin and eosin images were obtained using an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan). A photomicrograph of a DISH sample is shown in Fig. 1.

Staging and pathologic review

Primary breast cancer cases were staged according to the American Joint Committee on Cancer (AJCC) Staging Manual, 6th edition [34]. Tumors were graded according to the modified Black's nuclear grading system [35], and histologic classification was performed according to the World Health Organization criteria [36]. A patient was considered to have estrogen receptor (ER)- or progesterone receptor (PgR)-positive disease if there was at least 1% nuclear staining in the tumor. Hormone receptor-positive disease was defined as either ER- or PgR-positive disease.

HER2 status was obtained from the patients' medical records. HER2 status was defined as positive if IHC demonstrated a staining score of 3+ and/or FISH demonstrated a gene copy ratio of *HER2/CEP17* ≥ 2.0. Pathologic review was also carried out. Membrane staining in IHC was scored as 0, 1+, 2+, or 3+ according to the manufacturer's specifications (Dako, Carpentaria, CA, USA).

Table 1 Neoadjuvant chemotherapy regimen

Chemotherapy	Number of patients (*n*)
EC-DTX	12
EC-PTX	9
AC-PTX	2
PTX	2
Trastuzumab	*n*
With trastuzumab	5
Without trastuzumab	20

E: epirubicin, A: adriamycin, C: cyclophosphamide, PTX: paclitaxel, DTX: docetaxel

Statistical analysis

Means and standard deviations were calculated for age at diagnosis. Sensitivity and specificity were determined on the basis of the number of patients, not the number of lesions. McNemar's test was used to test the differences in the accuracy (sensitivity and specificity) between DISH and IHC. A p-value less than 0.05 was considered significant. All analyses were performed using the R software v 2.7.2 (http://www.r-project.org).

RESULTS

Among 46 patients, 8 patients (17.4%) achieved pCR. The residual tumor was sufficient to assess post-treatment HER2 status in 25 patients. DISH performed on pretreatment specimens confirmed HER2 amplification before beginning therapy (Fig. 2).

The characteristics of the patients are shown in Table 2. Pretreatment specimens were available for 25 patients. Regarding the ER status, 16 patients had ER- positive and 9 patients had ER- negative tumors. Meanwhile, 22 patients were diagnosed as HER2 positive on the basis of IHC 3+ and 3 patients were diagnosed as HER2 positive based on FISH results. Five patients received trastuzumab as neoadjuvant chemotherapy.

HER2 status assessed by DISH and IHC in pretreatment specimens is shown in Table 3. Of the 25 patients with HER2-positive tumors determined by IHC and/or FISH, 3 patients were diagnosed as HER2- negative by DISH. The sensitivity of the DISH and HER2 tests (i.e., IHC 3+ and/or FISH positive) was 88%. Of the 22 patients determined to be IHC 3+, 20 patients were determined to be DISH ≥ 2.0 and 2 patients were DISH

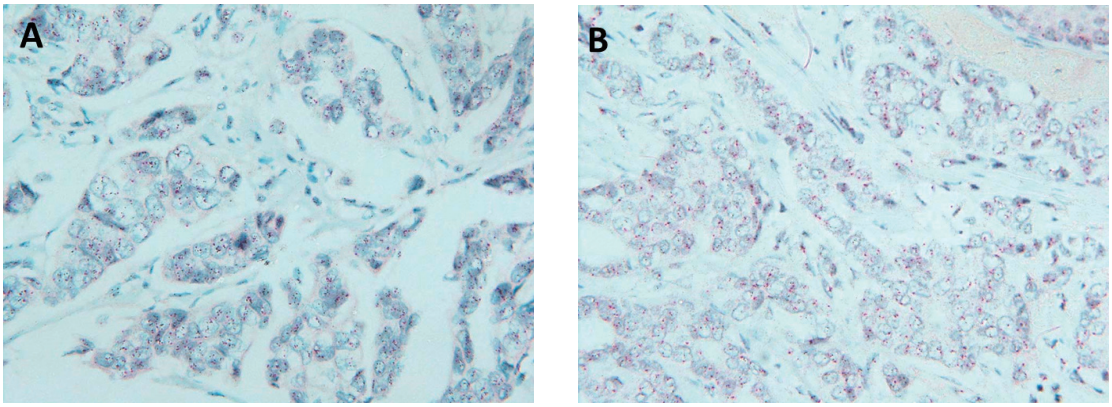

Fig. 1 DISH Images showing the following. (A) HER2 amplification, (B) HER2 without amplification (Black signal; HER2, red signal; CEP17)

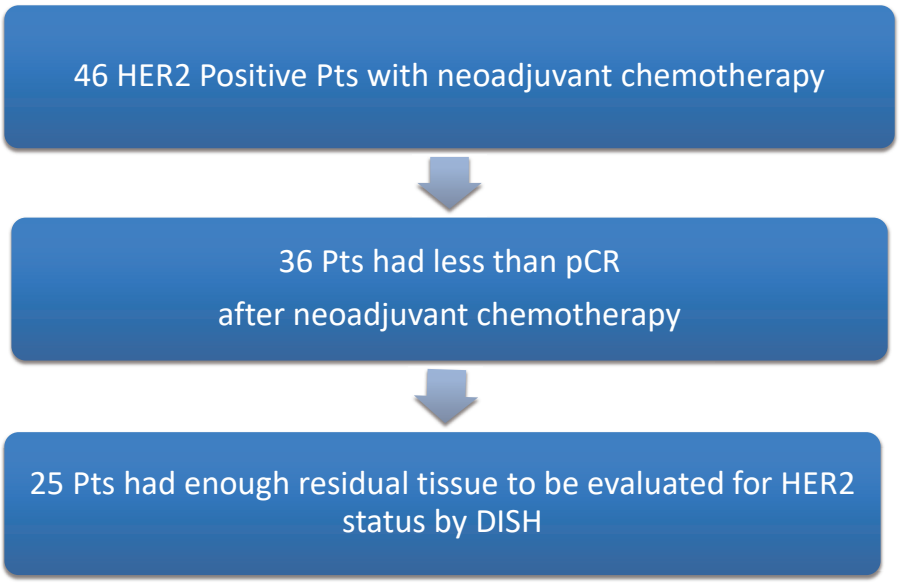

Fig. 2 Study scheme

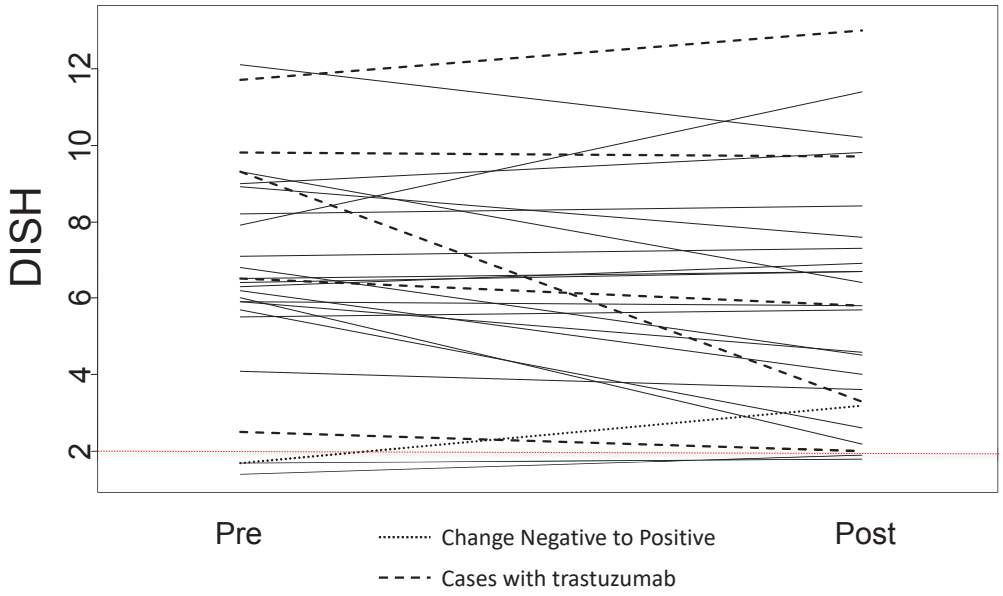

Fig. 3 HER2 amplification (HER2/CEP17 ratio) by DISH pre- and post-neoadjuvant therapy HER2 amplification by DISH pre- and post-neoadjuvant therapy is shown. No post-treatment specimens were found to be HER2 negative by DISH when compared to HER2-positive pretreatment tumors. Among 3 pretreatment tumors determined to be HER2 negative by DISH, 1 post-treatment tumor was found to be HER2 positive by DISH and 2 had stable HER2 status.

Table 2 Patient characteristics

Total number of patients (*n*)		*n* = 25
Age (years)	(Median)	54 (34–72)
T stage	1	0
	2	16
	3	6
	4	3
N stage	1	7
	2	15
	3	3
ER	Positive	16
	Negative	9
PgR	Positive	13
	Negative	12
HER2-IHC	3+	22
	2+ (FISH ≥ 2.0)	3
Neoadjuvant trastuzumab	Yes	5
	No	20

ER: Estrogen Receptor, PgR: Progesterone Receptor, IHC: Immunohistochemistry, FISH: Fluorescence in-situ hybridization, HER2: Human epidermal growth factor receptor-2

Table 3 Comparison of HER2 status by DISH and immunohistochemistry (IHC) in pre-treatment tumors

DISH/IHC	IHC 3+ (*n* = 22)	IHC 2+ (FISH ≥ 2.0) (*n* = 3)
DISH ≥ 2.0	20	2
DISH < 2.0	2	1

IHC: immunohistochemistry, HER2: Human epidermal growth factor receptor-2, DISH: Dual-color in situ hybridization, FISH: Fluorescence in-situ hybridization

< 2.0. Meanwhile, 3 patients were determined to be IHC 2+ and FISH ≥ 2.0, 2 patients were DISH ≥ 2.0, and 1 patient was DISH < 2.0.

HER2 amplification by DISH in pre- and post-neoadjuvant treated tumors is shown in Fig. 3. No post-treatment specimens were determined to be HER2 negative by DISH among pretreatment tumors determined to be HER2 positive by DISH. In 3 pre-treatment tumors determined to be HER2 negative by DISH, 1 post-treatment tumor was found to be HER2 positive by DISH and 2 had stable HER2 status.

DISCUSSION

The results demonstrate that HER2 status determined by DISH is stable between pretreatment breast tumors and residual tumors. However, the present results have few discrepancies compared to the previous studies. HER2 expression is reported to be unstable according to IHC in paired samples of pre- and post-treatment tissue from patients treated with trastuzumab in neoadjuvant setting. Burstein *et al.* [26] evaluated 23 paired sample pre- and post-neoadjuvant therapy with 12 weeks of paclitaxel and trastuzumab in patients with residual tumor by IHC to determine HER2 status. HER2 status changed from 3+ to 2+ in two patients and to 0 in four patients. Meanwhile, Harris *et al.* [27] report that among 48 patients treated with 12 weeks of neoadjuvant trastuzumab and vinorelbine, the HER2 conversion rate was 12% in 18 patients with sufficient residual tissue determined by IHC. Guarnei *et al.* [7] analyzed 40 and 67 HER2-positive patients treated with neoadjuvant chemotherapy without and with anti-HER2 agents, respectively. A loss of HER2 expression was observed in 40% and 14.7% of patients with residual disease after chemotherapy alone and chemotherapy plus anti-HER2 agents, respectively. Another study showed a loss of HER2 amplification in FISH in paired samples of pre- and post-treatment specimens from patients treated with neoadjuvant trastuzumab. Meanwhile, Hurley *et al.* [28] showed that 43% of tumors with HER2 gene amplification analyzed by FISH, before treatment with

neoadjuvant trastuzumab, docetaxel, and cisplatin became negative after therapy. Mittendorf *et al*. [29] reported that FISH performed on pretreatment specimens confirmed HER2 amplification before beginning therapy; whereas in post-treatment tumors, 8 of 25 patients (32.0%) were found to be HER2 negative by FISH. In our data, No post-treatment specimens were determined to be HER2 negative by DISH when compared to the corresponding pretreatment tumors that were determined to be HER2 positive by DISH. One possible reason for this discrepancy between the present and previous studies is that many patients in the previous studies received both trastuzumab and chemotherapy, whereas only 5 patients received trastuzumab and chemotherapy in the present study. Another possible reason for this discrepancy is that the previous studies used IHC or FISH to evaluate HER2 status, whereas the present study used DISH analysis.

Although HER2 overexpression detected by IHC is concordant with HER2 gene amplification by FISH [37–39], there are issues regarding consistency in IHC testing that may affect results, including antigen retrieval methods, fixation, and analysis or interpretations by the observer [40]. FISH is more reproducible between central and peripheral laboratories than IHC [39, 41]. The concordance between DISH and FISH for HER2 gene diagnosis was 96.2% in 134 invasive breast cancer patients [33]. Because the present study used DISH to determine HER2 gene amplification status in pre- and post-neoadjuvant chemotherapy, the changes in HER2 status are very unlikely to be due to artifacts or inconsistent testing.

We have previously reported that trastuzumab therapy is not associated with an increase in the loss of HER2 positivity in metastasis whereas chemotherapy is associated with an increase in the loss of such positivity [31]. Concordantly, among patients with residual disease treated within chemotherapy alone and chemotherapy plus anti-HER2, loss of HER2 was observed in 40% and 14.7% patients, respectively [7]. However, few studies have evaluated HER2 changes after neoadjuvant chemotherapy in both HER2-positive and HER2-negative patients and reported no significant changes in the HER2 rates [15, 42].

Nevertheless, it is unclear whether loss of HER2 amplification reflects response to therapy or a resistance mechanism and whether chemotherapy can promote clonal selection of *HER2/neu*-amplified cancers. Possible explanations include true biological change, treatment-induced clonal selection, pre-analytical and analytical pitfalls, sampling errors, and tumor heterogeneity [30]. It is unclear if patients with HER2-negative tumors after neoadjuvant chemotherapy should receive anti-HER2 treatment. This is because sampling by core needle biopsy in pretreatment settings is not representative of the character of the whole tumor. Discontinuing the drug in false-positive cases in core needle biopsy in pretreatment settings would avoid unnecessary treatment after loss of HER2 amplification after neoadjuvant therapy. However, in the event of a false-negative result of target expression in core needle biopsy in pretreatment settings, anti-HER2 treatment should be started after gain of HER2 amplification after neoadjuvant therapy.

The main limitation of this study is its retrospective nature, incurring a possibility of selection bias and precluding the determination of causal relationships. In addition, the sample size was relatively small, limiting the generalizability of the results.

In conclusion, HER2 status determined by DISH is stable between pretreatment breast tumors and residual tumors. However, there is a discrepancy in HER2 status determined by IHC and DISH. In addition, large prospective studies are required to clarify the concordance/discordance of HER2 status between pre- and post-neoadjuvant treatment; in particular, such studies should perform a central re-assessment of the HER2 status of both primary and metastatic lesions by using FISH or DISH in order to minimize the risk of inter-assay variability.

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