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DEVELOPMENT OF NOVEL ONE-STEP, FULLY AUTOMATED IN-SITU HYBRIDIZATION OF HER2 USING NON-CONTACT ALTERNATING CURRENT ELECTRIC FIELD MIXING

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Abstract
Human epidermal growth factor receptor 2 (HER2)-targeted agents are effective treatment for patients with HER2-positive breast cancer. However, the lack of survival benefit in HER2-negative patients as well as the toxic effects and high cost of these drugs highlight the need for accurate and prompt assessment of HER2 status. Our aim was to evaluate the clinical utility of a novel one-step, fully automated in-situ hybridization (Auto-RISH) method developed to facilitate hybridization. This method takes advantage of the non-contact mixing effect of an alternating current electric field. Eighteen specimens from patients diagnosed with primary breast cancer identified immunohistochemically as HER2 (2+) or (3+) were used. The specimens were all tested using DISH with an automated slide stainer (DISH-ASS), fluorescence in-situ hybridization, and Auto-RISH. With RISH the HER2 test was completed within 6 h, as compared to 20-22 h needed for the standard protocol. We found 88.8% agreement between standard DISH-ASS and Auto-RISH based on the status of the HER2 signals, and both methods stained nearly all samples from HER2-IHC 3+ patients equally well. These findings suggest Auto-RISH could potentially serve as an automated clinical tool for prompt determination of HER2 status in breast cancer samples.

Key words : breast cancer, HER2, in-situ hybridization

Introduction

Human epidermal growth factor receptor 2 (HER2 ; also referred to as ErbB2/neu) is a proto-oncogene located on the long arm of chromosome 17 and encodes a transmembrane receptor in the epidermal growth factor receptor (EGFR) family[1)]. Its overexpression is observed in approximately 15-25% of patients with breast cancer[2)], is associated with a poor prognosis, and is an indication for HER2 targeted therapy using trastuzumab, an anti-HER2 humanized monoclonal antibody[3,4)]. Combining trastuzumab with chemotherapy has markedly improved outcomes among patients with early HER2-positive breast cancer, reducing the risk of disease recurrence and death[5-7)]. In patients with HER2-positive metastatic breast cancer, pertuzumab plus trastuzumab and docetaxel improved both overall and progression-free survival as a new HER2 targeted therapy[8-10)]. In addition, patients

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treated using T-DM1 (trastuzumab conjugated to the microtubule inhibitor DM1) have a better prognosis than those treated with lapatinib and capecitabine[11]. These HER2-targeted therapies are an effective therapeutic approach for HER2-positive patients with invasive and metastatic breast cancer, but they are ineffective in HER2-negative patients. All breast cancers should therefore be accurately and promptly assessed for levels of HER2.

We have been developing a rapid immunohistochemistry (IHC) method that makes use of an alternating current (AC) electric field to facilitate the antigen-antibody reaction. We previously reported its usefulness for detection of lung cancer metastasis, breast cancer metastasis, central nervous system tumors and mammalian eggs, as well as in-situ hybridization (ISH)-HER2 and ISH-Echinoderm microtubule-associated protein-like 4 gene, and anaplastic lymphoma kinase gene (EML4-ALK) rearrangement in lung cancer[12-17]. With this device, we apply a high-voltage, low-frequency AC electric field to the sections. The resultant coulomb force stirs the antibody solution on the sections, thereby facilitating antibody binding. This rapid-IHC method enables prompt detection of target cells in frozen sections and can provide a surgeon with an intraoperative diagnosis within about 30 min[15-17]. In addition, the amount of HER2 antibody used is reduced with this IHC method, and there is less nonspecific staining[13]. We have shown that this technique is potentially applicable in multiple ways, for example in the hybridization step of dual ISH (DISH). With rapid DISH using non-contact AC electric mixing (RISH), HER2 tests can be completed within 6 h, as compared to 20-22 h needed for conventional DISH[18]. RISH thus has the potential for use as a clinical tool enabling prompt determination of HER2 status. However, RISH has a limitation : it is subject to human error because the ISH steps are manually applied ; only the hybridization step with AC mixing is automated. To address this limitation, we developed a new automated RISH (Auto-RISH), which reduces the effort of researchers or technicians and stabilizes the quality of HER2 tests. The aim of the present study was to evaluate the clinical utility and reliability of the new Auto-RISH technique.

Materials and Methods

Patients and Specimens.

All experimental protocols were approved by the institutional review board at Akita University Hospital (Permit number : 1,408), and written informed consent was obtained from all patients. The methods used in this study were carried out in accordance with the approved guidelines. Patients who had undergone needle biopsy and/or radical surgery were randomly enrolled in the study between 2013 and March 2018, and were analyzed retrospectively. The patients' clinical characteristics are listed in Table 1. Eighteen specimens from patients diagnosed with primary breast cancer were identified as HER2 0/(1+), (2+) or (3+) using IHC and were used in this study. Using standard histological techniques, the specimens were fixed in 10% formalin, embedded in paraffin, cut at 3-4 μm, transferred to slides, and stained using hematoxylin/eosin and IHC. In addition, the specimens were subjected DISH using an automated slide stainer (DISH-ASS) and our Auto-RISH protocol.

Standard Immunohistochemistry

IHC was performed using a BenchMark XT Staining

Table 1. Patient Characteristics

Number of patients		18			
			Hormone receptor status		
			ER	0	6
Age	Median	60.5±12.5		1+	0
	Range	42-84		2+	0
				3+	12
			PgR	0	9
Right		12		1+	1
Left		6		2+	5
				3+	3
Tumor size			HER-2 IHC	2+	9
	T0-1	14		3+	9
	T2	3			
	T3-4	1			
Lymph node					
	Negative	12			
	Positive	6			

Platform (Ventana Medical Systems, Tucson, AZ) with an automatic staining protocol. Standard IHC for HER2 was performed to decide whether to treat with trastuzumab. An anti-HER2/neu (4B5) rabbit monoclonal antibody (Roche Diagnostics, Penzberg, Germany) was used as the primary antibody with the appropriate dilution. Reacted antibodies were visualized enzymatically using brown diaminobenzidine as a substrate.

HER2 Scoring system

The intensity and pattern of membrane immunoreactivity were evaluated by two independent pathologists in accordance with the new recommendations of the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP)[19]. If the initial HER2 test of a core needle biopsy specimen from a primary breast cancer was negative, a new HER2 test was ordered for the excised specimen. We first tested for HER2 using a validated IHC assay. If the HER2 IHC test reported a score greater than HER2 (2+) as equivocal, we ordered an ISH using the same specimen.

Fluorescence ISH (FISH) to decide whether to use HER2 targeted therapy clinically

FISH for HER2 gene amplification was conducted using PathVysion HER2 DNA probe kits (Abbott Molecular Inc, Des Plaines, IL) at SRL Inc. Tokyo, Japan.

DISH-ASS

INFORM HER2 Dual ISH DNA Probe Assays were performed on a BenchMark XT Staining Platform (Ventana Medical Systems, Tucson, AZ). The run protocol was established so that the entire assay procedure, consisting of baking, deparaffinization, pretreatment, hybridization, stringency wash, signal detection and counterstaining, was completed as a one-step fully automated assay.

New automated rapid ISH (Auto RISH)

We have developed a prototype for Auto-RISH (Fig. 2). The theory behind and technique for AC electric field mixing was described in detail previously[14-16]. After mounting an insoluble label cover (SLS/E-bar rabel II, Roche Diagnostics, Penzberg, Germany) with a 1-cm diameter hole in the center on each microscope slide, 10 μl of ZytoDot 2C SPEC HER2/CEN 17 Probe (P) were applied evenly. Thereafter, 40 μl of HAIKORU-K140N (KANEDA, Japan), which is a very low viscosity liquid paraffin (4.0-5.5 mm^2/s), were added as an oil cover to prevent probe evaporation. The new devise automatically performs the denaturation and hybridization steps, anti-DIG/DNP step, AP/HRP step, AP chromogenic reaction step, HRP chromogenic reaction step, and slide washing step. The slide was placed between the electrodes and a high-voltage (4.5 kV, offset 2.4 kV), low-frequency (15 Hz) AC current was applied. There was a distance of 3.8-4.0 mm between the slide and electrode plates, and the current was applied for 3 h at 37°C. Anti-DIG/DNP mix (30 μl) and HRP/AP polymer mix (30 μl) were applied for 20 min under the same voltage and frequency conditions after the slide was covered with liquid paraffin.

Table 1 summarizes each procedure and the time required for ISH. The HER2 amplification based on the dual probe HER2/CEP 17 ratio with an average HER2 copy number and the signals/cell was evaluated according the ASCO/CAP scoring criteria[19] in both the DISH and RISH protocols.

Statistical analysis

Statistical analysis was performed using JMP IN 11.0.0 software (SAS Institute, Cary, NC, USA). Cohen's kappa-coefficient was used to assess agreement of 4×2-contingency tables between protocols. A kappa-value < 0.4 indicated fair to poor agreement, 0.4-0.8 indicated moderate to good agreement, and > 0.8 indicated excellent agreement.

Results

The clinical characteristics of the study participants are summarized in Table 1. Using the procedures outlined in Table 2, the new Auto RISH enabled detection of HER2 amplification within 6 h, as compared to 13.5 h needed for standard DISH-ASS. In the patients with HER2-IHC 3+, the performances of the new Auto RISH protocol and DISH-ASS were nearly equal, differing in only one sample (Table 3, Figure 1). In that specimen,

Table 2. Procedural details for DISH, DISH by automated slide stainer, and Auto-RISH

Protocol	Auto-RISH	DISH-ASS
Dewaxing, activation, dehydration	1 h	1 h
Denaturation and hybridization	+ 3 h (37°C) AC mixing	8 h (40°C)
Anti-DIG/DNP	+ 130 min Drop probe & antibodies	150 min
AP/HRP		
AP chromogenic reaction		
HRP chromogenic reaction		
Washing slides and other step		2 h
Total time	6 h	13.5 h

DIG, digoxigenin ; DNP, dintrophenyl ; AP, alkaline phosphatase : HRP, horseradish peroxidase ; AC, alternating current electric field ; ASS, Automated slide stainer

Table 3. Results of HER2 amplification based on one-step, fully automated RISH and DISH-ASS

HER2 IHC	Case	Auto-RISH		DISH-ASS	
3+	9	Negative	0	Negative	0
		Equivocal	0	Equivocal	0
		Positive	8	Positive	9
		Not evaluable	1	Not evaluable	0

ASS, Automated slide stainer
Auto-RISH, Automated AC mixing ISH

HER2 and CEP17 signals were not detected in the cell nucleus. In HER2-3+ IHC patients, we found 88.8% agreement between DISH-ASS and Auto-RISH based on the status of the HER2 signals (Cohen kappa coefficient was not calculated).

HER2 amplification in HER2 IHC (2+) samples using FISH, Auto RISH and standard DISH-ASS is summarized in Table 4. Cases 1, 2, 5, and 6 showed same result with each method. In case 4, FISH and Auto RISH were evaluated as negative, whereas DISH-ASS was evaluated as positive. In case 8, Auto RISH and DISH-ASS were evaluated as negative, while FISH was evaluated as positive (first FISH was equivocal, but the repeated test was evaluated as positive). In case 9, FISH was evaluated as

Figure 1. One-step, fully automated rapid ISH (Auto-RISH) device used to apply a high-voltage, low-frequency alternating current (AC) electric field.
The new devise automatically performs the denaturation and hybridization step, anti-DIG/DNP step, AP/HRP step, AP chromogenic reaction step, HRP chromogenic reaction step, and slide washing step.

Table 4. HER2 amplification of HER2-IHC (2+) samples using FISH, Auto-RISH and DISH-ASS

Case	FISH	Auto-RISH	DISH-ASS
1	+	+	+
2	−	−	−
3	−	Not evaluable	Not evaluable*
4	−	−	+
5	−	−	−
6	−	−	−
7	+	Not evaluable	+
8	+	−	−
9	−	Not evaluable	+

−, no stain ; +, amplification was evaluated by two independent pathologists in accordance with the new recommendations of the ASCO / CAP. *DISH-ASS could not be used because the paraffin-embedded tumor would not remain on the slide.

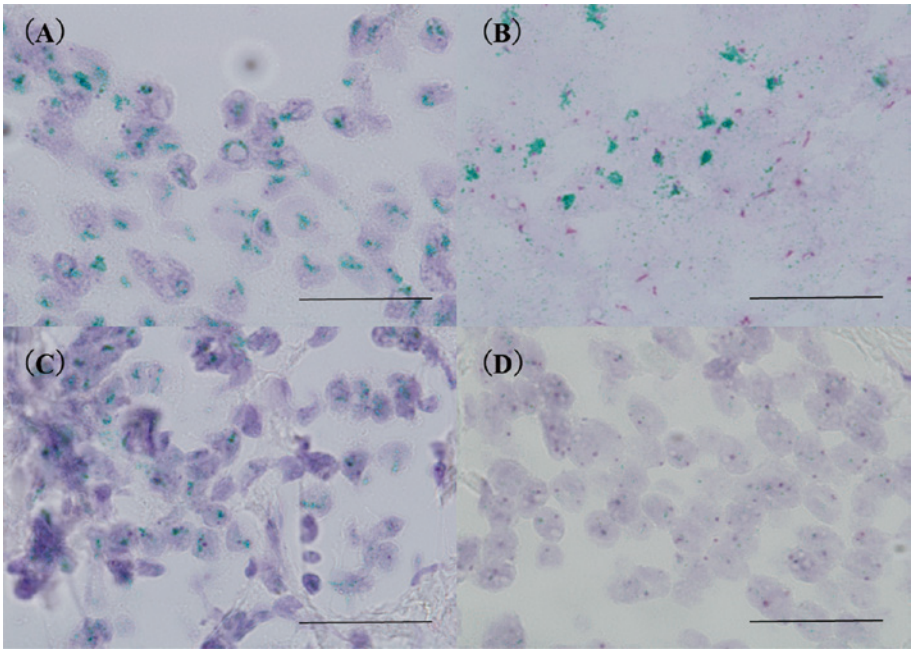

Figure 2. Detection of Breast Cancer Human Epidermal Growth Factor Receptor 2 (HER2) using a new automated AC mixing ISH (Auto-RISH).
(A) (B) Positivity based on ASCO/CAP scoring criteria demonstrated using Auto-RISH. (C) HER2 (2+) with IHC. Positivity demonstrated using Auto-RISH. (D) Negativity demonstrated using Auto-RISH. Red signals represent the chromosome 17 centromere ; green signals represent the HER2/neu gene locus at 17q12. Scale bar represents 50 μm.

negative, Auto RISH was not evaluable, and DISH-ASS was evaluated as positive. With all methods, no HER2 and/or CEP17 signals were observed in 3 specimens (cases 3, 7, and 9). For all samples, we found 66.6% agreement between DISH-ASS and Auto RISH based on the status of HER2 signals (Cohen kappa coefficient = 0.455, 95% confidence interval (CI) : 0.140-0.770).

Discussion

In the present study, we demonstrated that our new Auto RISH performed with a high-voltage, low-frequency AC electric field can be used to detect HER2 amplification within 6 h and can be completed as a one-step fully automated assay. The Auto-RISH and standard DISH-ASS stained nearly equally well in patients with HER2-IHC 3+ breast cancer. The high level of agreement between IHC and ISH means that neither is superior to the other as a predictor of the response to HER2-targeted treatment[2,20-22], though testing gene amplification using ISH is more reliable. However, the ISH protocol has several well-known disadvantages : it is time-consuming, technically demanding, costly, and may delay final determination of HER2 status. The standard ISH protocol requires approximately 22 h to complete, as hybridization usually takes 16-18 h. By contrast, RISH enabled the hybridization step to be completed in less than 3 h. However, because all the steps except hybridization in the RISH protocol were performed manually, the method was subject to human error and needed improvement. For this series, therefore, we developed a one-step, fully automated RISH device, which stabilized test quality.

The Cohen kappa coefficient 0.455 in the present study indicated moderate to good agreement between DISH and Auto-RICH, which is not sufficient for clinical

use. This reflects the technical challenges of ISH signal instability and scoring difficulties. Our new Auto-RISH system has some of the same limitations as ISH because it makes use of the ZytoDot 2C SPEC HER2/CEN 17 Probe set. However, Auto-RISH has a clear time advantage : DISH tests using Auto RISH were completed within 6 h, as compared to 20-22 h needed for conventional DISH.

The AC mixing device can also be applied to rapid ISH[12,18] and reagent-saving IHC[13]. However, ISH usually requires warm temperatures (37°C for hybridization overnight and 80°C for denaturation) and a longer processing time than the rapid IHC method. Consequently, evaporation can be a problem with the ISH protocol. We therefore developed and used a very low viscosity liquid paraffin as an oil cover when using the AC-IHC device in the present study.

In addition, because pores created when an electric current is applied to the cell membrane could cause false positives and over-diagnosis, it was important to consider the effect of electroporation when using the AC mixing device for IHC or ISH. In our earlier report, we showed that the electric current does not flow through the inside of microdroplets during AC electric field mixing[16]. This is because the stirred microdroplet is separated from the electrode by both glass and air, which act as insulators. Consequently, no pores are created.

In summary, we have presented a new one-step, fully automated ISH (Auto-RISH) technology that could potentially serve as a clinical tool for prompt determination of HER2 status in breast cancer samples. Although the present study is preliminary, with only a small sample and without an optimized HER2 RISH protocol, our findings indicate this approach warrants further investigation.

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Conflict of Interest Statement

The authors have no conflict of interest.

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