Quantitative assessment of HER2 gene amplification of breast cancer using droplet digital PCR

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INTRODUCTION

Human epithelial growth factor receptor 2 (HER2) is a member of the epidermal growth factor receptor family.\(^1\) HER2 gene amplification is the main mechanism of HER2 protein overexpression,\(^2\) and HER2 gene-amplified and/or HER2 protein-overexpressed cancer is determined as HER2-positive. At present, HER2 protein expression is generally assessed by immunohistochemistry (IHC). Although IHC is easy to perform, the results from different examinations can vary according to sample conditions or specific staining processes. HER2 gene copy number is detected using an in situ hybridization (ISH). ISH provides better diagnostic accuracy and added confidence, but it is more time-consuming, labor-intensive, and expensive compared to IHC.

To solve current problems of HER2 status diagnosis, we previously reported the usefulness of droplet digital polymerase chain reaction (ddPCR) for the assessment of HER2 gene amplification in breast cancer using formalin-fixed and paraffin-embedded sections.\(^3\) In our previous study, we calculated the HER2/CEP17 ratio (HER2 gene signals to chromosome 17 signals) with ddPCR and tumor content ratio (TCR) of each sample and determined the HER2 status by adopting a two-dimensional chart. This “ddPCR-TCR method” showed a high concordance with conventional HER2 status. In this study, we updated our method to assess the HER2 status of breast cancer in a more quantitative manner. We combined obtained data of the ddPCR method with tumor content ratio (TCR) [\(R_x\)] and the HER2/CEP17 ratio (estimated HER2/CEP17 ratio of a tumor cell)\(^4\). eHER2 was equivalent to conventional in situ hybridization (ISH) HER2/CEP17 ratio in most cases. eHER2 and ISH ratio showed a strong correlation (Spearman rank correlation \(\rho = 0.70, p < 0.0001\)). The obtained results indicated that eHER2 is a potential tool for HER2 status diagnosis in breast cancer.

Abstract

We previously reported the usefulness of droplet digital polymerase chain reaction (ddPCR) for the assessment of Human epithelial growth factor receptor 2 (HER2) gene amplification in breast cancer using formalin-fixed and paraffin-embedded sections. In our previous study, we combined HER2/CEP17 ratio (HER2 gene signals to chromosome 17 signals) with ddPCR and tumor content ratio (TCR) of each sample and determined the HER2 status by adopting a two-dimensional chart. This “ddPCR-TCR method” showed a high concordance with conventional HER2 status. In this study, we updated our method to assess the HER2 status of breast cancer in a more quantitative manner. We combined obtained data of the ddPCR method with tumor content ratio (TCR) [\(R_x\)] and the HER2/CEP17 ratio (estimated HER2/CEP17 ratio of a tumor cell)\(^4\). eHER2 was equivalent to conventional in situ hybridization (ISH) HER2/CEP17 ratio in most cases. eHER2 and ISH ratio showed a strong correlation (Spearman rank correlation \(\rho = 0.70, p < 0.0001\)). The obtained results indicated that eHER2 is a potential tool for HER2 status diagnosis in breast cancer.

KEYWORDS

breast neoplasms, diagnostic technics and procedures, gene amplification, HER2 genes, polymerase chain reaction

Abbreviations: ASCO/CAP, American Society of Clinical Oncology/College of American Pathologists; CEP17, chromosome 17 centromere; DCIS, ductal carcinoma in situ; DISH, differentiation induction subtraction hybridization; ddPCR, droplet digital PCR; eHER2, estimated HER2/CEP17 ratio of a tumor cell; FFPE, formalin-fixed, paraffin-embedded; FISH, fluorescence in situ hybridization; HER2, human epithelial growth factor receptor 2; IHC, immunohistochemistry; ISH, in situ hybridization; PCR, polymerase chain reaction; TCR, tumor content ratio.

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chromosome 17 signals) with ddPCR and the tumor content ratio (TCR) with a digital slide scanner for each sample. The calculated values were plotted on a two-dimensional chart, named ddPCR-TCR chart: samples plotted above the cut-off line was determined as HER2 positive; those plotted below the line as HER2 negative. This method of HER2-status determination, named “ddPCR-TCR method,” showed a high concordance with conventional approaches to evaluate HER2 status. Furthermore, we not only succeeded in automating a large part of the process from DNA extraction to determination of HER2 status but also reduced the costs compared with conventional HER2 examinations.3

A limitation of our previous method was that the way of evaluating HER2 status was more qualitative than quantitative because of the necessity of a two-dimensional chart. In this study, we updated the ddPCR-TCR method intending to assess HER2 status in breast cancer in a more quantitative manner. We statistically analyzed the obtained data and examined whether our new strategy is applicable for clinical use.

**METHODS**

**Patient cohort**

FFPE samples were collected from 41 primary breast cancer patients, the same cohort as our previous study,3 who underwent surgery at The University of Tokyo Hospital from 2009 to 2011 (Table 1). Patients with ductal carcinoma in situ (DCIS), a tumor size of < 1 cm, and history of preceding chemotherapies were excluded. Tumor sizes ranged from 10 to 43 mm. All samples included in the study were reassessed for their HER2 status according to the latest version of American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines.4 A tumor was considered positive for HER2 when the IHC scoring result was 3+ positive or 2+ with a CEP17 ratio of < 2.0 and average HER2 gene copy of < 4.0 signals/cell determined using either fluorescence in situ hybridization (FISH) or differentiation induction subtractions hybridization (DISH), counting at least 20 cells within the area. A tumor was considered negative for HER2 when the IHC scoring result was 0, 1+, or 2+ with a HER2/CEP17 ratio of < 2.0 and average HER2 gene copy of < 4.0 signals/cell determined byISH. This study had no ISH equivocal cases (HER2/CEP17 ratio < 2.0; average HER2 gene copy ≥ 4.0 and < 6.0 signals/cell). Among 10 HER2 3+ cases, four cases were determined as HER2 2+ at the time of diagnosis according to firstly published guidelines.2 Finally, 15 patients were diagnosed as positive for HER2.

**ddPCR and TCR calculation**

The data of ddPCR ratio of HER2/CEP17 and TCR were obtained in our previous report.3 Briefly, DNA was extracted from FFPE sections of the samples and purified using the MagCore Genomic DNA FFPE One-Step Kit following the manufacturer’s instructions (Cartridge Code, 405; running time, 2 h; elution volume, 60 μL). Neither macro- nor micro-dissection were performed before DNA extraction from FFPE samples. ddPCR was performed on a QX200 droplet digital PCR system (Bio-Rad) with HER2 primers (ERBB2-13F: CCGTGACTTTCCCTGATGCT, ERBB2-13R: GCCATGGAGGCCCTACATT, and ERBB2-13P: FAM/TGAGTCTCA/ZEN/AGATCTC/31ABkFQ) and CEP17 primers (ch17cent-6F: TCATTCCTGCAGCCCTTTG, CEP17 cent-6R: GCCATGACGAATTAACGCG, and ch17cent-6P: VIC/AGCGAGCTCC/ZEN/AGCCCA/31ABkFQ) (Integrated DNA Technologies, Coralville, IA, USA). PCRs were performed in a total volume of 20 μL containing 10 μL Bio-Rad 2× ddPCR Supermix for Probes (no dUTP), HER2 primers (500 nM ERBB2-13F, 500 nM ERBB2-13R, and 250 nM ERBB2-13P), CEP17 primers (500 nM ch17cent-6F, 500 nM ch17cent-6R, and 250 nM ch17cent-6P), 10–260 ng DNA, and water. The reaction mixtures were partitioned into an emulsion of approximately 20,000 droplets in oil using a QX200 Droplet Generator. After performing PCR, the PCR plate was loaded on a Bio-Rad QX200 droplet

**TABLE 1 Patients’ clinicopathological characteristics**

| Age (years) median 60.0 (range 28–85) | (n) |
|--------------------------------------|-----|
| < 50                                 | 10  |
| ≥ 50                                 | 31  |
| Tumor size (mm) (range 10–43 mm)     |     |
| 10 ≤, < 20                           | 21  |
| 20 ≤, < 30                           | 8   |
| 30 ≤                                 | 12  |
| Estrogen receptor expression         |     |
| Negative                              | 9   |
| Positive                              | 32  |
| Lymph node metastasis                |     |
| Negative                              | 25  |
| Positive                              | 16  |
| Stage                                |     |
| 1                                     | 17  |
| 2A                                    | 14  |
| 2B                                    | 9   |
| 3A, B                                | 0   |
| 3C                                    | 1   |
| 4                                     | 0   |
| Treated with trastuzumab              |     |
| Yes                                   | 8   |
| No                                    | 33  |
reader and read using Bio-Rad QuantaSoft version 1.6.6 software. Fluorescence amplitude thresholds to discriminate the positive droplets from the negative droplets were set at 1500 for FAM channel (HER2) and 3000 for VIC channel (CEP17). The HER2/CEP17 copy number ratio was analyzed by calculating the copies per droplet from the Poisson distribution.5

The TCRs were calculated as the number of AE1/AE3-positive (1:200, Leica Biosystems) cells within the tumor region divided by the number of all detected cells in FFPE tissue sections. We used Definiens Tissue Studio (ver 3.6) to count stained and unstained cells separately.6–8 The invasive tumor areas were manually enclosed as regions of interest (ROI), while non-invasive tumors surrounding the invasive tumors were excluded.

**Estimated HER2/CEP17 ratio of a tumor cell (eHER2)**

In our previous report,3 a two-dimensional “ddPCR-TCR chart” was used to determine HER2 gene amplification. In this chart, the vertical axis represents the ddPCR ratio \( R \) \((0 < R)\) and the horizontal axis represents the TCR \( X \) \((0 < X \leq 1)\). If there are exactly twice as many HER2 genes as CEP17 in a tumor cell, the HER2/CEP17 ratio obtained by ddPCR \( R_x \) can be expressed as \( x + 1 \) (Figure 1a). If one of the samples is plotted above the theoretical cut-off line \( x + 1 \), the cancer cells in this sample are considered to have HER2 gene amplification \((HER2/CEP17 ratio over 2.0)\) (Figure 1b).

In the current study, we developed a new strategy to assess HER2 status in a more quantitative
way by combining data of ddPCR ratio and TCR. Suppose there is a sample in which the HER2/CEP17 ratio in tumor cells (R₁) is unknown, but its ddPCR ratio [Rₓ] and TCR [x] are successfully obtained; Rₓ can then be expressed as “(R₁ – 1) x + 1.” Thus, R₁, the unknown HER2/CEP17 ratio, can be calculated from the following formula: (Rₓ – 1)/x + 1 (0 < x ≤ 1). We named this HER2/CEP17 ratio “eR₁” calculated using ddPCR ratio [Rₓ] and TCR [x] as “eHER2” (estimated HER2/CEP17 ratio of a tumor cell) (Figure 1c).

Figure 1d is a graphical concept of eHER2. The obtained TCR (x) bears a proportionate relationship to (Rₓ–1), so (Rₓ–1)/x is equal to (R₁–1)/1. Hence eHER2 (=R₁) can be expressed as (Rₓ – 1)/x + 1. Alternatively, eHER2 (=R₁) can be calculated by plugging “X = 1” into R = x⁻¹X + 1, a straight line connecting two points, (X, R) = (0,1) and (x, Rₓ), on the graph. In the current study, eHER2 was calculated for all the samples, and these values were compared to the obtained conventional ISH ratios.

**Setting equivocal range**

Although ddPCR analysis is an exact way to detect HER2 gene amplification, errors in the small range are inevitable due to the manual process of preparing reagent solutions. In establishing the ddPCR-TCR method in our previous report, we set the equivocal area on the chart obtained by cell line assay. In establishing the ddPCR-TCR method in our previous report, we set the equivocal area on the chart that we obtained by cell line assay. To increase the sensitivity, we adopted the range of 1.8–2.2 as an equivocal range of eHER2 (Figure 2a), based on the equivocal range for FISH assays of HER2 gene amplification in the firstly published version of ASCO/CAP guidelines. This means that a sample with eHER2 between the range 1.8–2.2, or whose Rₓ is between 0.8x + 1 and 1.2x + 1, has the possibility of either HER2 gene twice-amplified and not.

There is another problem that also may decrease the accuracy of eHER2. When the TCR of a sample is

![Diagram](image-url)

**FIGURE 2** Newly designed equivocal ranges. (a) The range 1.8–2.2 was adopted as the equivocal range of eHER2 based on the equivocal range for in situ hybridization (ISH) assays of HER2 gene amplification in the former version of ASCO/CAP guidelines. A sample with eHER2 between the range 1.8–2.2, or whose Rₓ is between 0.8x + 1 and 1.2x + 1, has both possibility of HER2-gene twice-amplified and not. (b) The equivocal area in (a) is rotated and fit on droplet digital polymerase chain reaction - tumor content ratio (ddPCR-TCR) chart. This second equivocal area indicates that the determination of HER2 status by calculating eHER2 can be misleading if a sample is plotted in it, or in other words, if Rₓ of the sample is between 0.8x + 0.8 and 0.8x + 1.2. (c) When applying these equivocal areas from (a,b) on ddPCR-TCR chart with the clinical cases plotted, these areas almost overlap the previous equivocal area (shaded in blue). Also, cases within the previous equivocal area (cases 3, 4, 8, 22, 27, 32, 33, and 35 in Table 2) are all included in the newly combined equivocal areas.
high enough, the effect of the measuring error of ddPCR analysis on eHER2 calculation is not so large. In contrast, samples with very low TCR could result in an overestimated or underestimated eHER2. To overcome this problem, we set one more equivocal range to try to increase the specificity of our method. We rotated the equivocal area in Figure 2a and fit it on ddPCR-TCR chart (Figure 2b). This second equivocal area indicates that the determination of HER2 status by calculating eHER2 can be misleading if a sample is plotted in this area, or in other words, if R2 of the sample is between 1.2x+0.8 and 0.8x+1.2.

When applying these equivocal areas on the ddPCR-TCR chart with the clinical cases plotted, these areas almost overlap the previous equivocal area. Also, cases within the previous equivocal area (cases 3, 4, 8, 22, 27, 32, 33, and 35 in Table 2) are all included in the newly combined equivocal areas (Figure 2c).

**Statistical analysis**

Statistical analysis was performed using R (version 4.0.3). A non-parametric Spearman rank order correlation was used to determine the relationship between ISH ratio and eHER2. Receiver operating characteristic (ROC) curve was generated to evaluate the ability of eHER2 to discriminate HER2 positive case versus HER2 negative case. The Kaplan–Meier method was employed to analyze the rates of recurrence-free survival (RFS) and overall survival (OS) among patients. The log-rank test or log-rank test for trend were used to compare the patients determined to be HER2 negative, equivocal, or positive, based on eHER2 results. p value of less than 0.05 was defined as statistically significant.

**RESULTS**

**Correlation between conventional HER2 status and eHER2**

The data of conventional HER2 status (IHC score and ISH ratio), ddPCR ratio, TCR, and eHER2 for all 41 breast cancer samples are summarized in Table 2.

Correlation between HER2 IHC score and eHER2, ISH ration and eHER2, and HER2 status according to ASCO 2018 and eHER2 are shown in Figure 2a–c. There were significant differences for eHER2 between IHC 0, 1+ to 2+ (p = 0.040), 2+ to 3+ (p = 0.003), and 0, 1+ to 3+ (p = 0.0016) (Figure 3a). Also, eHER2 was significantly higher in ISH ratio ≥2.0 cases compared to ISH ratio < 2.0 cases (p < 0.001) (Figure 3b). When diagnosed according to ASCO 2018 guidelines, HER2...
positive cases showed significantly higher eHER2 than HER2 negative cases ($p < 0.001$) (Figure 3c). ISH ratio and eHER2 showed a very strong correlation (Spearman rank correlation, $\rho = 0.70; p < 0.0001$).

According to the ROC curve analysis, a cut-off value = 1.67 of eHER2 was determined (sensitivity and specificity, 100% and 88%, respectively, $p < 0.0001$), and area under the curve was 0.982 (Figure 3d).

**HER2/CEP17 ratio of ISH and eHER2 for each sample**

Figure 4 shows the *HER2/CEP17* ratio of ISH (blue bars) and eHER2 determinations (orange bars) for each sample. The theoretical cut-off value of 2.0 is depicted as dotted line, and one of the equivocal ranges 1.8–2.2 is expressed as a gray zone on the chart. Cases within either or both of the two newly established equivocal ranges are marked with asterisks, and their case numbers (Table 2) are indicated on the chart. All cases with an ISH ratio $\geq 2.0$ showed a high eHER2, at least over 1.8. There were two

### TABLE 2 (Continued)

| Case number | HER2 IHC score (ASCO 2018) | HER2 IHC score (ASCO 2007) | HER2 ISH ratio | ddPCR ratio | TCR | eHER2 |
|-------------|---------------------------|---------------------------|----------------|-------------|-----|-------|
| 37          | 3+                        | 6.84                      | 2.25           | 0.595       | 3.10|
| 38          | 3+                        | 6.90                      | 3.52           | 0.681       | 4.70|
| 39          | 3+                        | 9.11                      | 4.77           | 0.300       | 13.57|
| 40          | 3+                        | 8.32                      | 5.15           | 0.491       | 9.45|
| 41          | 3+                        | 10.0                      | 13.8           | 0.629       | 21.35|

Abbreviations: ASCO, American Society of Clinical Oncology; ddPCR, droplet digital polymerase chain reaction; eHER2, estimated HER2/CEP17 ratio of a tumor cell; IHC, immunohistochemistry; ISH, in situ hybridization; TCR tumor content ratio.
discrepant cases (cases 4 and 36) in which the ISH ratio was less than 1.8 but eHER2 was over 2.2. Of these, case 4 was included within the equivocal ranges, but case 36 was outside of the ranges.

Overall survival and recurrence-free survival analysis of the patients

To investigate the association of eHER2 with the patients’ survival outcome, we analyzed the OS and RFS of 41 patients. In the all-patient analysis, survival time was not significantly different among HER2 negative, equivocal and positive patients determined by eHER2 (Log-rank test for trend, $p = 0.66$ in OS; $p = 0.54$ in RFS). When there is a lymph node metastasis, patients with positive HER2 status tended to have worse RFS, though not significantly, than those with a negative or equivocal (log-rank test for trend, $p = 0.081$) (Figure 5). When grouping HER2 negative and equivocal patients together, RFS was significantly worse in HER2 positive patients than in others (log-rank test, $p = 0.040$). No statistical difference was demonstrated among three groups of eHER2 when dividing the patients with other factors such as estrogen-receptor positivity, pathological stages, and trastuzumab-treatment (Figure S1).

DISCUSSION

In this study, we improved our method by applying a very simple but potent strategy to quantitatively assess HER2 status at the final step of the ddPCR-TCR method. We calculated the unknown ratio of HER2 gene to CEP17 in only tumor cells by combining the ddPCR ratio and TCR and named this calculated HER2/CEP17 ratio of tumor cells as eHER2. ISH ratio and eHER2 showed high correlation, and our results indicated that eHER2 is equivalent to ISH ratio. With our method, clinicians and pathologists will be able to obtain the data for the HER2/CEP17 ratio in breast cancers easily and cheaply without the technical needs or costs of performing ISH.

Although ddPCR is a very precise device for DNA amplification measurement, this method has a measuring error to some extent, and this makes it difficult to determine the HER2 status of cases with eHER2 only slightly higher or lower than 2.0. To evaluate such cases with caution, we set the equivocal range as $1.8 \leq eHER2 \leq 2.2$, or $0.8x + 1 \leq R_x \leq 1.2x + 1$, based on the determination of HER2 gene amplification with ISH in the first ASCO/CAP guidelines for breast cancer. One factor that hinders the accuracy ddPCR analysis is the heterogeneity of HER2 expression and/or HER2 gene amplification within a tumor. Wang et al. evaluated HER2 gene amplification with ddPCR, specifically for the HER2...
equivocal cases, and showed high accuracy and usefulness of ddPCR. Still, they also considered that the presence of intratumoral heterogeneity of HER2 would make the evaluation challenging. Therefore, it seems reasonable to set the equivocal range in our method. Such cases within this range may well be determined their HER2 status comprehensively considering the results of conventional tools such as IHC and ISH. However, we speculate that this range could be set much narrower, considering the potential of ddPCR.

The effect of the error also depends on the TCR of each case; the influence of error is relatively small if the
TCR of a specimen is high enough, while the range of the error of calculated eHER2 can be larger as TCR gets smaller. Based on this observation, we set another equivocal range: $1.2x + 0.8 \leq R_e \leq 0.8x + 1.2$. This second equivocal range is graphically symmetrical about a point with the first equivocal range (Figure 2).

The eHER2 of cases within the second equivocal range can be calculated as far away from the true HER2/CEP17 of tumor cells, so we need to be careful when determining the HER2 status of a case. The eHER2 of cases with very low TCR but out of the second equivocal range may also be far from the true HER2/CEP17 but is still useful in HER2 status determination. For example, a case with relatively low TCR (e.g., 0.2) but with very high ddPCR ratio (e.g., over 2.0) would be HER2 positive without doubt, and vice versa.

The presence of DCIS lesions within the tumor might also cause the eHER2 calculation to go wrong. As there were only a small amount of DCIS components in our cases, we excluded DCIS lesions surrounding the invasive tumors from ROI in this study. However, those tumors with a predominant intraductal component and significant discrepancy of HER2 expression between invasive and non-invasive tumor cells can mislead the HER2 status determination in our method. In the current guidelines, HER2 expression is to be measured at the invasive components. Also, anti-HER2 therapy is generally indicated for cases with a tumor diameter of more than 1 cm but not for smaller tumors or HER2-positive DCIS. In our method, it can be supposed that the larger the invasive lesion is, the smaller the DCIS component's effect becomes; thus, selecting the adequate cases for ddPCR-TCR method is essential to determine the indications for anti-HER2 therapy properly. Even if there are some borderline samples, our equivocal range will play a crucial role in reducing HER2 status misjudgment.

Most of the cases, except for cases within the equivocal ranges, showed strong concordance between conventional ISH ratio and our established eHER2, however one case (case number 36 in Table 2 and Figure 4) showed discrepant results between the two. In this case, the ISH ratio was lower than 1.8 while eHER2 was higher than 2.2. HER2 gene amplification was calculated with FISH at the time of diagnosis and was no longer observable because of degraded fluorescence, so we re-examined this sample with DISH. Five slides were granted to evaluate the quality of the DISH exam, and we found instability of CEP17 signals among the slides, indicating that the quality of FFPE specimen may be defective owing to insufficient fixation or degradation of the tissue. The re-examined result of the HER2/CEP17 ratio with DISH was 1.56, which was similar to the value using FISH (1.70), but these ratios may be underestimated. The IHC staining results from this case also showed some discrepancies. One pathologist diagnosed this case as HER2 with an IHC score 3+, because complete circumferential membrane staining could be seen for at least 10% of tumor cells, but another pathologist diagnosed as HER2 score of 2+ since membrane staining was not sufficient to determine as completely circumferential. These discrepant diagnoses of IHC between the two pathologists are likely owing to the low quality of the specimen. With our method using ddPCR, the HER2/CEP17 ratio can be measured accurately unless the DNA of tumor cells is severely damaged, and at this point our method is still advantageous over conventional assays. Clinically, this patient did not receive anti-HER2 therapy after surgery, but fortunately no sign of recurrence has been seen in 10 years and regular outpatient follow-up for this patient has been completed.

We focused on quantifying HER2 status determination and established the calculated value of eHER2 to simplify the evaluation for clinicians and pathologists to recognize the degree of HER2 gene amplification. However, our final goal is to use eHER2 as both a predictive factor of anti-HER2 therapy and a prognostic factor. The direct target of currently marketed anti-HER2 drugs is indeed overexpressed-HER2 protein, not amplified-HER2 gene. However, ASCO/CAP guidelines treat IHC and ISH equally as methods for HER2 test in breast cancer; thus, we developed a technique to digitally assess HER2 gene amplification as a novel HER2 testing tool, which makes better use of the characteristics of ddPCR. In our patient survival analysis, HER2 positive cases determined by eHER2 showed significantly worse RFS than non-positive patients when there is a lymph node metastasis. This result means eHER2 has the potential as a predictive marker for patients' survival, especially in cases with advanced breast cancer. In a past report, Xu et al. evaluated the association between disease-free survival and HER2 gene amplification level by ISH in a meta-analysis; however, they concluded that HER2 gene amplification level is not a prognostic factor for HER2-positive breast cancer with trastuzumab-based targeted therapy. We could not find a large-scale study that indicates the relationship between the rate of HER2/CEP17 with ISH and the effectiveness of anti-HER2 antibody. Also, there has been no report showing the relationship between HER2 status examined with ddPCR and the therapeutic effect of anti-HER2 therapy in breast cancer, including the assessment of pathological complete response rates after preoperative chemotherapy. Additional studies including more cases are required, and future research should analyze whether eHER2 is a useful predictive or prognostic marker for HER2-positive breast cancer patients.

In conclusion, we evolved the strategy for the ddPCR-TCR method from our previous report and proposed eHER2 as a new HER2 determination tool in breast cancer. We demonstrated that eHER2 has the
potential to replace the conventional HER2 examination methods. A larger scale study is needed to apply our method in clinical use.

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ETHICS APPROVAL
This study was carried out with permissions from the University of Tokyo Hospital Ethics Committee (Approved No. 11031-1)). All methods presented here were performed in accordance with the relevant guidelines and regulations approved by Faculty of Medicine, the University of Tokyo.

CONSENT TO PARTICIPATE
Written informed consent was obtained from all participants.

CONSENT FOR PUBLICATION
Patients signed informed consent regarding their data.

CONFLICT OF INTERESTS
None declared.

AUTHOR CONTRIBUTIONS
Kazutaka Otsuji and Takeshi Sasaki conceived and designed the study. Takeshi Sasaki, Masahiko Tanabe, and Yasuyuki Seto directed the study and supervised the research. Kazutaka Otsuji collected tumor specimens. Kazutaka Otsuji and Takeshi Sasaki confirmed histopathology findings and interpreted the clinical data. Kazutaka Otsuji performed droplet digital PCR analyses. Kazutaka Otsuji drafted the manuscript, with the assistance and final approval of all authors.

DATA AVAILABILITY STATEMENT
All data supporting the study are available on request. No proprietary materials except patient tissues were used.

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SUPPORTING INFORMATION
Additional Supporting Information may be found online in the supporting information tab for this article.

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