Quantitative Assessment of HER2 Gene Amplification of Breast Cancer Using Droplet Digital PCR

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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 ratio [R_x] and TCR [x]; we calculated “$(R_x - 1) / x + 1$” for 41 samples with primary breast cancer and named the value led by this formula as “*eHER2* (estimated *HER2*/CEP17 ratio of a tumor cell)”. *eHER2* was equivalent to conventional ISH *HER2*/CEP17 ratio in most cases. *eHER2* and ISH ratio showed a very 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.

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. HER2 is positive in 10%-25% of human breast cancers, and its positivity is associated with aggressive metastatic disease and a poor prognosis. However, since trastuzumab, an anti-HER2 antibody, became available in daily clinical practice, the prognosis of HER2-positive breast cancer has improved dramatically [1, 2]. 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. Thus, it is often challenging to make consistent classification of HER2 status with IHC. *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 (FFPE) sections [3]. ddPCR has gained attention as a more precise and less subjective method to quantify DNA amplification [4]. In our previous study, we calculated the *HER2*/CEP17 ratio (*HER2* gene signals to 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*, a tumor size of < 1 cm, and history of preceding chemotherapies were excluded. The median age of the patients was 60.0 years (range, 28–85 years). Tumor sizes ranged from 10 to 43 mm. All samples included in the study had been previously assessed for their HER2 status according to the previous version of American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines [5]. A tumor was considered positive for HER2 when the IHC scoring result was score 3 + positive (circumferential membrane staining that is complete, intense, and in > 10% of tumor cells) or 2+ (circumferential membrane staining that is incomplete and/or weak/moderate and in > 10% of tumor cells or complete and circumferential membrane staining that is intense and in ≤ 10% of tumor cells) with a *HER2*/CEP17 ratio of ≥ 2.0 or average *HER2* gene copy of ≥ 6.0 signals/cell determined using either fluorescence *in situ* hybridization (FISH) or differentiation induction subtraction hybridization (DISH), counting at least 20 cells within the area. A tumor was considered negative for HER2 when the IHC scoring result was score 0 (no membrane staining), 1+ (faint or barely perceptible/incomplete membrane staining), or 2 + with a *HER2*/CEP17 ratio of < 2.0 and average *HER2* gene copy of < 4.0 signals/cell determined by ISH. 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 not performed before DNA extraction from FFPE samples. ddPCR was performed on a QX200 droplet digital PCR system (Bio-Rad) with HER2 primers (ERBB2-13F: CCCTCCGTACTTCCTGATGCT, ERBB2-13R: GCCATGGAGAGCCTCACATT, and ERBB2-13P: FAM/TGAGAGTCA/ZEN/AGATCTC/3IABkFQ) and CEP17 primers (ch17cent-6F:

CGCTCCTGCACTGTAACACGT, ch17cent-6R: TCATTCCTGCAGCCCTTGA, and ch17cent-6P: VIC/AGCAGGTCC/ZEN/AGCCCA/3IABkFQ) (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 reader and read using Bio-Rad QuantaSoft version 1.6.6 software. The *HER2*/CEP17 copy number ratio was analyzed by calculating the copies per droplet from the Poisson distribution [6].

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; Munich, Germany) to count stained and unstained cells separately [7–9].

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] ($0 < X \leq 1$). An FFPE section contains both tumor and non-tumor cells, and thus the extracted DNA is derived from both kinds of cells. 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 $x+1$ (Fig. 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) (Fig. 1b). In determining the HER2 statuses of the clinical samples, instead of setting a clear cut-off line, we settled an equivocal area on the chart that we calculated in a cell line assay.

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 (R_1) is unknown, but its ddPCR ratio [R_x] and TCR [x] are successfully obtained; R_x can then be expressed as “$(R_1 - 1)\ x+1$”. Thus, R_1, the unknown *HER2*/CEP17 ratio, can be calculated from the following formula: $(R_x - 1) / x+1$ ($0 < x \leq 1$). We named this *HER2*/CEP17 ratio “R_1” calculated using ddPCR ratio [R_x] and TCR [x] as “*eHER2*” (estimated *HER2*/CEP17 ratio of a tumor cell) (Fig. 1c).

Figure 1d is a graphical concept of *eHER2*. The obtained TCR (x) bears a proportionate relationship to ($R_x - 1$), so $(R_x - 1) / x$ is equal to $(R_1 - 1) / 1$. Hence *eHER2* (= R_1) can be expressed as $(R_x - 1) / x+1$.

Alternatively, *eHER2* (= R_1) can be calculated by plugging “$X = 1$” into $R = \frac{Rx - 1}{x} X + 1$, a straight line connecting two points, (X, R) = (0, 1) and (x, R_x), 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

In establishing the ddPCR-TCR method in our previous report [3], we set the equivocal area on the chart that we obtained by cell line assay. In this assay, we extracted genomic DNA from two cell lines: one cell line assuming *HER2* gene double-amplified tumor cells, and another cell line assuming *HER2* non-amplified normal cells. We mixed the DNA in a stepwise manner to construct TCR patterns from 10–90%, analyzed the samples using ddPCR three times, and plotted the results on a ddPCR-TCR chart.

In the assay, the linear regression line obtained from the plotted data was very close to our supposed cut-off line "$R = X + 1$", which successfully confirmed our theory of the ddPCR-TCR method. However, we noticed that there were measuring errors using ddPCR analysis, even though it was a very small range. We assumed these errors were due to the manual process of preparing reagent solutions before analyzing gene amplification by ddPCR. These observations suggest that if we analyze the *HER2*/CEP17 ratio by ddPCR and calculate TCR of a sample containing tumor cells with double-amplified *HER2* gene, the *eHER2* might not be exactly 2.0 every time. The previous and ongoing ASCO/CAP guideline [5, 10] sets ISH positivity as *HER2*/CEP17 ratio ≥ 2.0, so the cut-off value of *eHER2* may theoretically be 2.0. However, determining cases with *eHER2* only slightly less than 2.0 as "HER2 negative" would be risky.

To increase the sensitivity, we adopted the range of 1.8–2.2 as an equivocal range of *eHER2* (Fig. 2a), based on the equivocal range for FISH assays of *HER2* gene amplification in the firstly published version of ASCO/CAP guidelines [2]. This means that a sample with *eHER2* between the range 1.8 to 2.2, or whose R_x is between $0.8x + 1$ and $1.2x + 1$, has the possibility of either *HER2* gene twice-amplified and not.

However, there is another problem that also may decrease the accuracy of *eHER2*. When the TCR of a sample is 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 Fig. 2a and fit it on ddPCR-TCR chart (Fig. 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 R_x 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 [3], 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 on Table 2) are all included in the newly combined equivocal areas (Fig. 2c).

Statistical analysis

Statistical analysis was performed using JMP Pro statistical software (ver. 12.2.0, SAS Institute, Japan). 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.

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 2013 [5] and *eHER2* are shown in Fig. 2a, b and 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+ ($P = 0.0016$) (Fig. 3a). Also, *eHER2* was significantly higher in ISH ratio ≥ 2.0 cases compared to ISH ratio < 2.0 cases ($P < 0.001$) (Fig. 3b). When diagnosed according to ASCO 2013 guidelines [5], HER2 positive cases showed significant higher *eHER2* than HER2 negative cases ($P < 0.001$) (Fig. 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 (Fig. 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 ≥ 2.0 showed a high *eHER2*, at least over 1.8. There were two 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 equivocal ranges, but case 36 was out of the ranges.

Discussion

In this study, we reaffirmed the potential of the usefulness of ddPCR for the assessment of *HER2* gene amplification in breast cancer.

We developed the ddPCR-TCR method in our previous report and showed that *HER2* gene amplification assessing in breast cancer using ddPCR was feasible for clinical use [3]. However, in ddPCR-TCR method, the determination of *HER2* gene amplification in a tumor was based on the area on the ddPCR-TCR chart on which the case was plotted, making the assessment more qualitative than quantitative.

In the current 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 [R] and TCR [*X*] and named this calculated *HER2*/CEP17 ratio of tumor cells as *eHER2*. This new approach provides us information on *HER2* gene amplification of breast cancer specimens using a very simple and cost-effective method with ddPCR.

Our method has several advantages compared with the standard evaluation techniques. IHC and ISH are subjective in determining HER2 status, and special training is necessary for professional pathologists to make an accurate judgment. However, even pathology specialists often have difficulty in judging some equivocal or borderline cases. Our method can obtain accurate and precise data of *HER2* gene amplification of a tumor objectively and rapidly, which means that our method can relieve pathologists of a great burden. ddPCR is easy to perform, with only a few manual processes, and the running cost is far cheaper than ISH analyses. ISH is routinely performed for breast cancer specimens in some countries, but not all areas, mainly because of economic reasons. Our method using ddPCR provide information of *HER2* gene amplification for breast cancer cases with very low costs, which would help reduce medical spending.

The calculation of *eHER2* is the ultimate tool in our method. *eHER2* is the estimated *HER2*/CEP17 ratio of tumor cells using the obtained data of ddPCR ratio and TCR. In this study, we showed a high correlation between ISH ratio and *eHER2*, 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 [2]. 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 [11]. 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 TCR of a specimen is high enough, while range of the error of calculated *eHER2* can be larger as TCR gets smaller. Based on this observation, we set another equivocal range: $1.2x + 0.8 \leq R_x \leq 0.8x + 1.2$. This second equivocal range is graphically symmetrical about a point with the first equivocal range (Fig. 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.

By setting these two equivocal ranges, we succeeded in improving sensitivity and specificity of our method. The ranges were quite similar to the equivocal area on ddPCR-TCR method in our previous study [3], and eight cases within the previous equivocal area were also included in our new equivocal ranges. One of the goals of our method is to accurately select HER2-positive cases and thus to determine appropriate candidates of anti-HER2 therapy. To increase the precision of our method, we need to determine more optical equivocal ranges in the future.

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 Fig. 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 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 deficit 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, *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 over these six years.

Our method represents a novel strategy to evaluate HER2 status. Although this technique may be unusual for pathologists, who are trained to determine HER2 status by observing specimen, clinicians can easily integrate *eHER2* into daily medical practice, as are trained in examinations that yield numerical values and ranges, such as blood tests. However, while IHC and ISH can be visually observed repeatedly and are preserved with prepared slides, the calculated ddPCR ratio, TCR and *eHER2* are only calculated data, and these data must be managed carefully to avoid problems, such as patient mix-ups.

In this study, we focused on quantifying HER2 status determination and established the calculated value of *eHER2*, which has the potential to replace conventional HER2 examinations. One of the purposes of quantification in determining HER2 status is 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 predictive factor of anti-HER2 therapy and a prognostic factor. Xu *et al*. evaluated the association between disease-free survival and HER2 amplification level by ISH in a meta-analysis but concluded that *HER2* amplification level is not a prognostic factor for HER2-positive breast cancer with trastuzumab-based targeted therapy [12]. 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. The direct target of currently marketed anti-HER2 drugs is indeed overexpressed-HER2 protein, not amplified-*HER2* gene. However, ASCO/CAP guidelines [2, 5, 10] 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 the future, it would be necessary to select cases that are preferable to receive intensive anti-HER2 therapy, e.g. trastuzumab plus pertuzumab, in the adjuvant setting for early HER2-positive breast cancer, and *eHER2* may play an important role in these cases. Additional studies including more cases are required, and future research should analyze whether *eHER2* is a really 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 succeeded in showing a strong correlation between ISH ratio *(HER2* gene to CEP17) and *eHER2*, and 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.

Declarations

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.

Competing interests

All authors of this work declare that they have no conflict of interest.

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Authors' contributions

K.O. and T.S. conceived the study. T.S., M.T. and Y.S. directed the study and supervised the research. K.O. collected tumor specimens. K.O. and T.S. confirmed histopathology findings and interpreted the clinical data. K.O. performed droplet digital PCR analyses. K.O. wrote the manuscript, with the assistance and final approval of all authors.

Availability of data and material

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

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Tables

Due to technical limitations, table 1 and 2 is only available as a download in the Supplemental Files section.