Breast Cancer Res Treat (2017) 162:11–18
DOI 10.1007/s10549-016-4092-5

PRECLINICAL STUDY

Use of droplet digital PCR for quantitative and automatic analysis of the HER2 status in breast cancer patients

Kazutaka Otsuji[1] · Takeshi Sasaki[2] · Atsushi Tanaka[2] · Akiko Kunita[2] · Masako Ikemura[2] · Keisuke Matsusaka[2,3] · Keiichiro Tada[1] · Masashi Fukayama[2] · Yasuyuki Seto[1,4]

Received: 15 December 2016 / Accepted: 19 December 2016 / Published online: 30 December 2016

Abstract
Purpose Digital polymerase chain reaction (dPCR) has been used to yield an absolute measure of nucleic acid concentrations. Recently, a new method referred to as droplet digital PCR (ddPCR) has gained attention as a more precise and less subjective assay to quantify DNA amplification. We demonstrated the usefulness of ddPCR to determine *HER2* gene amplification of breast cancer.
Methods In this study, we used ddPCR to measure the *HER2* gene copy number in clinical formalin-fixed paraffin-embedded samples of 41 primary breast cancer patients. To improve the accuracy of ddPCR analysis, we also estimated the tumor content ratio (TCR) for each sample.
Results Our determination method for *HER2* gene amplification using the ddPCR ratio (*ERBB2*:*ch17cent* copy number ratio) combined with the TCR showed high consistency with the conventionally defined *HER2* gene status according to ASCO-CAP (American Society of Clinical Oncology/College of American Pathologists) guidelines ($P<0.0001$, Fisher's exact test). The equivocal area was established by adopting 99% confidence intervals obtained by cell line assays, which made it possible to identify all conventionally HER2-positive cases with our method. In addition, we succeeded in automating a major part of the process from DNA extraction to determination of *HER2* gene status.
Conclusions The introduction of ddPCR to determine the *HER2* gene status in breast cancer is feasible for use in clinical practice and might complement or even replace conventional methods of examination in the future.

Keywords Breast cancer · HER2 · Digital PCR · Gene amplification · Tumor content ratio

Introduction

Human epithelial growth factor receptor 2 (HER2; also known as ERBB2) is a member of the epidermal growth factor receptor family and the target of anti-HER2 antibodies such as trastuzumab. It is amplified and/or overexpressed in 10–25% of human breast cancers and is associated with aggressive metastatic disease and a poor prognosis [1]. In general, HER2 expression is assessed by immunohistochemistry (IHC), and the *HER2* gene copy number is detected using an in situ hybridization (ISH) assay. IHC is easier to perform, but the result can change if the sample condition or staining process is different, thus making classification of the HER2 status subjective. ISH is often performed when the HER2 status is classified as equivocal by IHC, and provides better diagnostic accuracy and added confidence, but it is more time consuming, labor intensive, and expensive than IHC.

Digital polymerase chain reaction (dPCR) was developed to yield an absolute measure of nucleic acid concentrations by the combination of limiting dilution, endpoint PCR, and Poisson statistics [2]. Quantitative PCR (qPCR), or real-time PCR, is also commonly used to

✉ Takeshi Sasaki
takesasa@m.u-tokyo.ac.jp

[1] Department of Breast and Endocrine Surgery, Faculty of Medicine, University of Tokyo, Tokyo, Japan

[2] Department of Pathology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan

[3] Department of Molecular Oncology, Graduate School of Medicine, Chiba University, Chiba, Japan

[4] Department of Stomach and Esophageal Surgery, Faculty of Medicine, University of Tokyo, Tokyo, Japan

quantitate nucleic acids, but some studies have shown that the precision and sensitivity of dPCR to resolve copy number changes are higher than those of qPCR [3]. Recently, a new method called droplet digital PCR (ddPCR) has gained attention as a more precise and less subjective assay to quantify DNA amplification [4]. ddPCR is a further improved method to perform dPCR, which is based on water–oil emulsion droplet technology. In ddPCR, a sample is fractionated into 20,000 droplets, and PCR amplification of the template molecules occurs in each individual droplet [5]. ddPCR has also been shown to obtain a high level of partitioning at a low cost [4].

Although ddPCR is a precise and robust method, the results can be affected by the conditions of tissue specimens from cancer patients, because tissue samples contain both cancer cells and non-cancer cells such as stromal cells and lymphocytes. To obtain more accurate data, ratios of tumor cells in tissue specimens should be considered when performing ddPCR analysis.

In this study, we used ddPCR to measure the *HER2* gene copy number in clinical formalin-fixed, paraffin-embedded (FFPE) samples from breast cancer patients, and concurrently calculated the tumor content ratio (TCR) in each sample. We developed a chart, which has been successfully applied to measure the HER2 copy number in gastric cancer [6], to determine *HER2* gene amplification while taking into consideration the ddPCR ratio combined with the TCR and analyzed the concordance between our determination method and the conventional method. Ultimately, we automated a major part of the process and verified the clinical utility of ddPCR.

Materials and methods

Patient cohort

FFPE samples were collected from 41 primary breast cancer patients 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 American Society of Clinical Oncology/College of American Pathologists (ASCO–CAP) guidelines [7]. A tumor was considered positive for HER2 when the HercepTest showed a 3+ positive result (circumferential membrane staining that is complete, intense, and in >10% of tumor cells) or 2+ positive result (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 as negative for HER2 when the HercepTest result score was 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. We had no ISH equivocal cases (*HER2*/*CEP17* ratio: <2.0; average *HER2* gene copy: ≥4.0 and <6.0 signals/cell) in this study. Among 10 HER2 3+ cases, four cases were determined as HER2 2+ at the time of diagnosis according to previously published guidelines [8]. Finally, 15 patients were diagnosed as positive for HER2 (Table 2).

Table 1 Clinicopathological characteristics of patients included in the study ($N = 41$)

	(*N*)
Age (years) median 60.0 (range 28-85)	
<50	10
≥50	31
Tumor size (mm) (range 10–43)	
<10	0
10≤, <20	21
20≤, <30	8
30≤	12
HER2 score	
0	4
1+	4
2+ (ISH−)	18
2+ (ISH+)	5
3+	10
Lymph node metastasis	
Negative	25
Positive	16
pStage	
1	17
2A	14
2B	9
3A, B	0
3C	1
4	0

ISH, in situ hybridization; *pStage,* pathological stage

DNA extraction

For each sample, DNA was prepared from one to three 5 μm-thick sections. DNA was extracted and purified

Table 2 Information on the HER2 status, ddPCR ratio and TCR

Case number	HER2 IHC score (ASCO 2013)	HER2 IHC score (ASCO 2007)	HER2 ISH ratio	ddPCR ratio	TCR
1	0		1.73	0.8	0.109
2	0		1.49	0.92	0.343
3	*0*		*1.26*	*1.03*	*0.147*
4	*0*		*1.12*	*1.22*	*0.172*
5	1+		0.74	0.532	0.615
6	1+		1.57	0.86	0.504
7	1+		1.1	0.91	0.611
8	*1+*		*1.86*	*1.34*	*0.434*
9	2+		1.1	1.14	0.72
10	2+		1.11	0.75	0.452
11	2+		1.13	1.24	0.528
12	2+		1.15	1.22	0.464
13	2+		1.18	1.19	0.354
14	2+		1.24	0.943	0.474
15	2+		1.26	1.25	0.484
16	2+		1.28	0.94	0.458
17	2+		1.3	1.12	0.658
18	2+		1.36	1.109	0.664
19	2+		1.38	1.22	0.626
20	2+		1.43	1.08	0.525
21	2+		1.64	1.2	0.718
22	*2+*		*1.65*	*1.66*	*0.606*
23	2+		1.69	1.2	0.371
24	2+		1.69	1.31	0.57
25	2+		1.72	1.24	0.529
26	2+		1.74	1.22	0.505
27	*2+*		*2.09*	*1.33*	*0.274*
28	2+		2.17	1.46	0.292
29	2+		2.74	1.97	0.568
30	2+		4.28	2.5	0.553
31	2+		4.83	2.23	0.306
32	*3+*	*2+*	*1.28*	*1.23*	*0.341*
33	*3+*	*2+*	*1.3*	*1.19*	*0.171*
34	3+	2+	4.06	3.74	0.341
35	*3+*	*2+*	*6.47*	*1.39*	*0.404*
36	3+		1.7	1.51	0.242
37	3+		6.84	2.25	0.595
38	3+		6.9	3.52	0.681
39	3+		9.11	4.77	0.3
40	3+		8.32	5.15	0.491
41	3+		10	13.8	0.629

Italics applied to #3, 4, 8, 22, 27, 32, 33, and 35 are consistent with the symbol colors in Fig. 3

IHC, immunohistochemistry; *ISH*, in situ hybridization; *ASCO*, American Society of Clinical Oncology; *ddPCR*, droplet digital polymerase chain reaction; *TCR*, tumor content ratio

automatically using a MagCore® Genomic DNA FFPE One-Step Kit following the manufacturer's instructions (Cartridge Code: 405; running time: 2 h; elution volume: 60 μL). The purified DNA was quantified using a NanoDrop 2000 spectrophotometer with 0.6–12.0 μg DNA recovered per section.

dPCR

dPCR was performed as described previously [9] on a QX200 droplet digital PCR system (Bio-Rad) with *ERBB2* primers (*ERBB2*-13F: CCCTCCGTACTTCCTGATGCT, *ERBB2*-13R: GCCATGGAGAGCCTCACATT, and *ERBB2*-13P: FAM/TGAGAGTCA/ZEN/AGATCTC/3IABkFQ) and *ch17-cent* primers (*ch17cent*-6F: CGCTCCTGCACTGTAACAC-GT, *ch17cent*-6R: TCATTCCTGCAGCCCTTGA, and *ch17-cent*-6P: VIC/AGCAGGTCC/ZEN/AGCCCA/3IABkFQ) (Integrated DNA Technologies, Coralville, IA, USA). MDA-MB361 DNA was used as a positive control. The PCRs were performed in a total volume of 20 µL containing 10 µL Bio-Rad 2× ddPCR Supermix for Probes (No dUTP), *ERBB2* primers (500 nM *ERBB2*-13F, 500 nM *ERBB2*-13R, and 250 nM *ERBB2*-13P), *ch17cent* 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. The droplets were then transferred to a 96-well PCR plate, heat sealed, and placed in a thermal cycler (Bio-Rad PX1). The thermal cycling conditions were 95 °C for 10 min, 40 cycles of 95 °C for 30 s and 56 °C for 60 s, 98 °C for 10 min, and a 4.0 °C hold. After the 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 *ERBB2*:*ch17cent* copy number ratio was analyzed by calculating the copies per droplet from the Poisson distribution [10]. We aimed for at least 500 droplets to be tested positive for *ch17cent* and at least 10,000 droplets accepted for whole counting to assess the ratio accurately.

TCR

To calculate the TCR, we first performed immunostaining with AE1/AE3 (1:200, Leica Biosystems) for each specimen. Then whole sections were scanned using a Nano-Zoomer 2.0-HT Digital slide scanner (Hamamatsu Photonics KK). Data were imported into Definiens Tissue Studio (ver 3.6; Munich, Germany) to count stained and unstained cells separately [11–13]. TCRs were calculated as the number of AE1/AE3-positive cells within the tumor region divided by the number of all detected cells in FFPE tissue sections.

ddPCR–TCR chart

A two-dimensional chart was applied to determine *HER2* gene amplification (Fig. 1a). In this chart, the vertical axis represents the ddPCR ratio [R] ($0 < R$) and the horizontal axis represents the TCR [x] ($0 \le x \le 1$). If there are exactly twice as many *HER2* genes as *CEP17* in a cancer cell, the *HER2*/*CEP17* ratio obtained by ddPCR [R] can be expressed as $x + 1$, based on the assumption that gene copy numbers in cancer cells are homogeneous and that non-cancer cells are genetically stable with diploid chromosomes [6] (Fig. 1b). When one of the samples is plotted above the cut-off line $x + 1$, the cancer cells in this sample are supposed to have amplified *HER2* (*HER2*/*CEP17* ratio over 2.0).

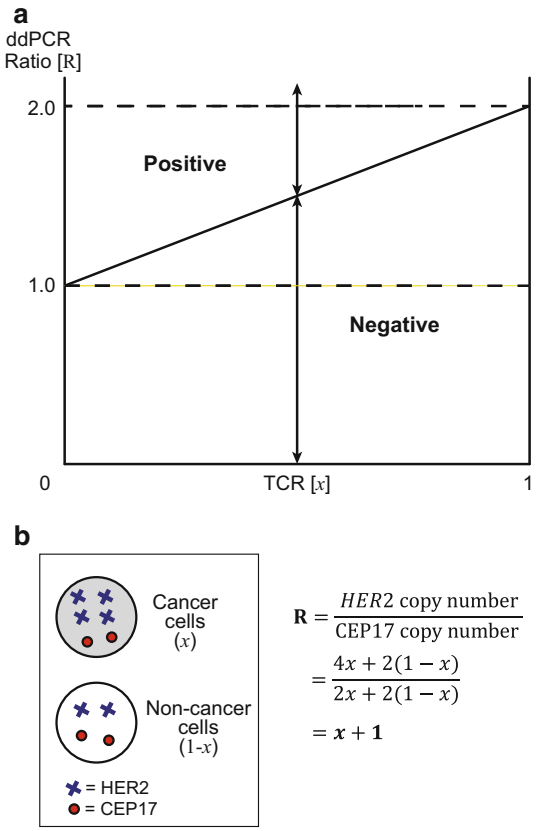

Fig. 1 Concept of the ddPCR–TCR chart. **a** Two-dimensional chart to determine *HER2* gene amplification. The vertical axis represents the ddPCR ratio [R] ($0 < R$). The horizontal axis represents the TCR [x] ($0 \le x \le 1$). **b** When there are exactly twice as many *HER2* genes as *CEP17* in a cancer cell, the *HER2*/*CEP17* ratio obtained by ddPCR [R] can be expressed as $x + 1$. We consider the straight line represented by [$R = x+1$] as the cut-off line. When a case is plotted above the cut-off line [$R = x+1$], the *HER2* gene in the cancer cells is determined as "amplified." When plotted below the cut-off line, the *HER2* gene is determined as "not-amplified"

Cell line assay

To confirm our ddPCR–TCR method, we mixed genomic DNA of two cell lines, constructed hypothetical TCR patterns, and analyzed them by ddPCR [6]. One cell line was the H522 lung cancer cell line obtained from the American Type Culture Collection (Manassas, VA, USA), assuming tumor cells with a double-amplified *HER2* gene. The other was the GM18997 Epstein–Barr virus-transformed lymphoblastoid

cell line (LCL) (Coriell Biorepository), assuming normal cells without *HER2* gene amplification. H522 cells were cultured in Dulbecco's modified Eagle's medium (Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin–streptomycin solution (Nacalai Tesque). LCL cells were cultured in Roswell Park Memorial Institute 1640 medium (Nacalai Tesque) containing 10% fetal bovine serum and 1% penicillin–streptomycin solution. Cells were cultured at 37 °C in an atmosphere containing 5% CO_2. DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA).

First, we analyzed the genomic DNA of the cell lines and determined the target gene copy number [copies/μL]. Then, we mixed the DNA in a stepwise manner to construct TCR patterns from 10 to 90%, and analyzed them by ddPCR three times. The results were plotted on a ddPCR–TCR chart. Linear regression analysis was performed to analyze the relationship between the TCR and ddPCR ratio in cell line assay, and 99% confidence intervals (CI) were calculated. On ddPCR–TCR chart, we determined HER2 as positive when a case is plotted above the 99% CI area, equivocal when plotted within the CI, and negative when plotted below the CI. We refer to this determination method as the ddPCR–TCR method.

Statistical analysis

All analyses were performed using JMP Pro statistical software (ver. 12.2.0, SAS Institute, Japan).

Results

ddPCR analyses were all performed successfully. AE1/AE3 staining was performed well, and there was no problem with calculations of the TCR by Definiens Tissue Studio.

The ddPCR ratio of the mixture of cell lines was plotted on a ddPCR–TCR chart (Fig. 2). The regression line obtained from the plotted data is expressed as $R = 1.0724x + 0.9862$, which is very close to our supposed cut-off line $R = x+1$, as shown in Fig. 1b. Ninety-nine percent CI is depicted in the chart.

The ddPCR ratio and TCR of each clinical case were plotted on a ddPCR–TCR chart (Fig. 3). On the chart, we applied a 99% CI in the cell line analysis as described above, and defined it as the "equivocal area." Eleven cases were plotted on the positive area: seven conventional HER2 3+ case, and four cases determined as 2+ in IHC and positive in ISH; 22 cases were plotted on the negative area: five HER2 0 and 1+ cases, and 17 cases determined as 2+ in IHC and negative in ISH. There were eight cases

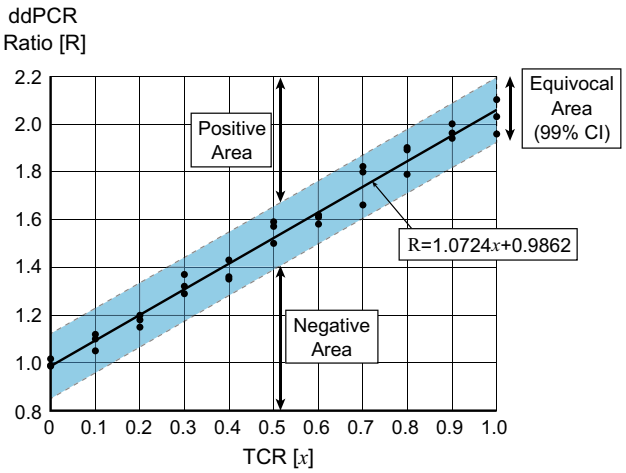

Fig. 2 Theoretical TCRs of cell lines. Genomic DNA of LCL and H522 cells was mixed in a stepwise manner and analyzed by ddPCR. The horizontal axis represents the ratio of H522 to LCL cells, corresponding to the TCR [x] in clinical cases. The vertical axis represents the ratio of ERBB2 to CEP17 in ddPCR [R]. The regression line obtained from plotted data was expressed as $R = 1.0724x + 0.9862$, which was very close to our supposed cut-off line $R = x+1$. The 99% CI (aqua) is depicted on the chart. We determined HER2 as positive when a case was plotted above the 99% CI area, equivocal when plotted within the CI, and negative when plotted below the CI. We refer to this determination method as the ddPCR–TCR method

plotted on the equivocal area: three HER2 3+ cases, one HER2 2+ (ISH positive) case, one HER2 2+ (ISH negative) case, and three HER2 0 and 1+ cases. As a whole, the sensitivity was 100% (15/15: cases plotted on positive and equivocal areas) and the specificity was 88.5% (23/26), showing high consistency between the conventional status and ours ($P < 0.0001$, Fisher's exact test). Information on the HER2 status, ddPCR ratio, and TCR is summarized in Table 2.

Discussion

We demonstrated a positive correlation between the results of our ddPCR–TCR method and the conventional HER2 status in samples from breast cancer patients. Our method also showed high sensitivity and specificity in determining *HER2* gene amplification. The HER2 status is currently judged visually by pathology experts, which is not only subjective but often hard and stressful work. In this study, we developed a more objective method using ddPCR, a new quantification assay for DNA amplification. With the aim of applying our method to clinical use, we automated the process, from DNA extraction to HER2 determination, as much as possible.

In this study, we extracted DNA from FFPE sections of surgical specimens. To collect as many tumor cells as

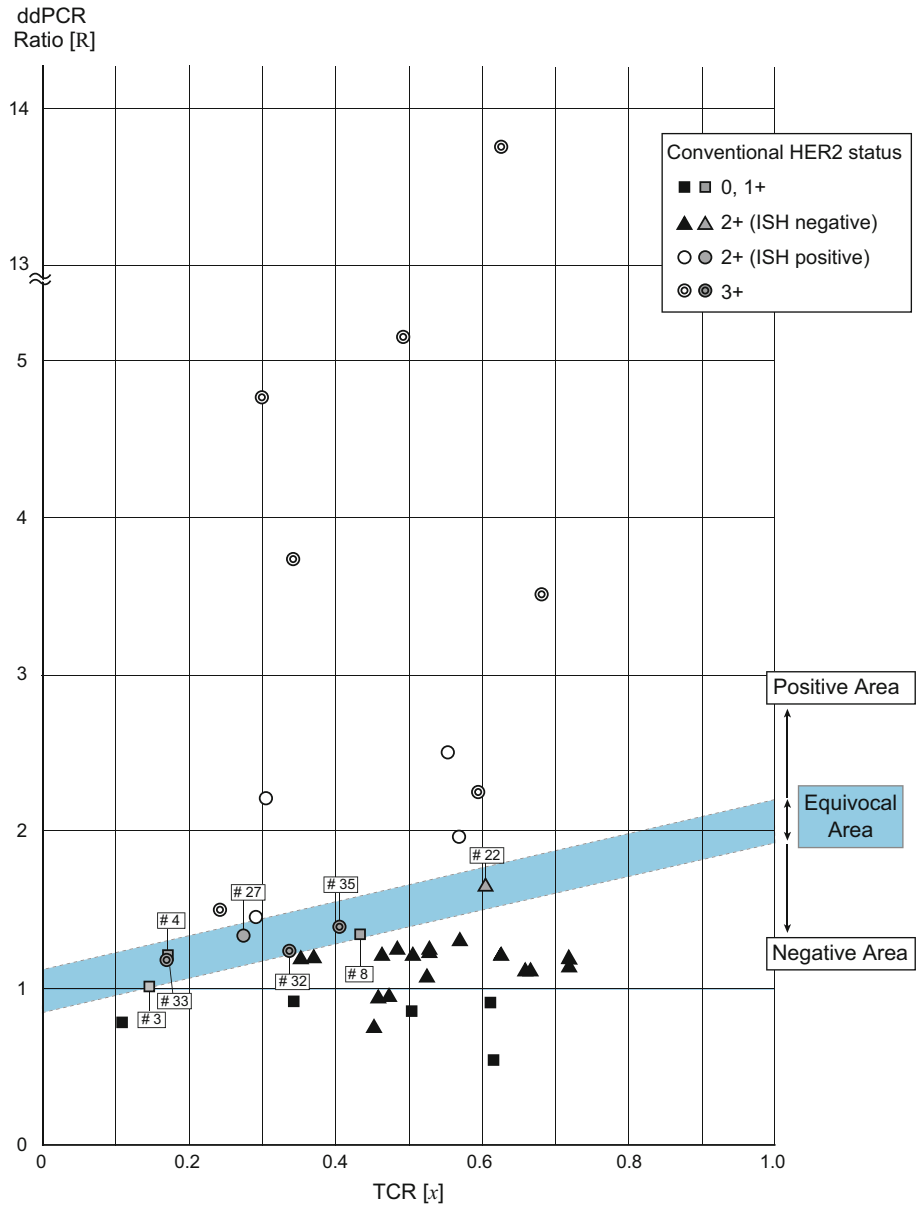

Fig. 3 Results of the 41 clinical cases depicted on the ddPCR–TCR chart. Findings of the conventional HER2 status are expressed as follows: IHC 0, 1+ (negative) as a *square*; IHC 2+ (equivocal) and ISH negative as a *triangle*; IHC 2+ (equivocal) and ISH positive as a *circle*; IHC 3+ (positive) as a *double circle*. Symbols of HER2-negative cases determined by conventional methods plotted on the negative area (below the 99% CI) are *black*, while symbols of conventionally HER2-positive cases on the negative area (above the 99% CI) are *white*. There are eight cases plotted on the equivocal area (symbols are *gray*, and patient numbers are shown)

possible, we selected samples with a tumor size of more than 1 cm. Non-cancer cells contained in each section had a significant effect on the ddPCR ratio of the *HER2* gene in samples with a relatively small amount of tumor cells. Garcia-Murillas et al. [10] also assessed *HER2* gene amplification in breast cancer patients, and their results were in high concordance with the conventional HER2 status according to ASCO–CAP guidelines (2007) [8]: 100% sensitivity (18/18) and 98% specificity (57/58). To achieve >70% tumor DNA content, they performed microdissection prior to DNA extraction. However, although microdissection is a good method to collect tumor cells, it is quite difficult to perform in daily clinical practice.

We overcame this limitation by estimating the TCR for each sample and assessed HER2 gene amplification by two parameters, the ddPCR ratio and TCR, using a two-dimensional chart. Definiens Tissue Studio® is a very useful software that calculates accurate TCRs by counting hundreds of thousands of cells automatically within a few hours. By considering the TCR for each sample, we can assess the ddPCR results of samples with a small tumor cell content and omit cumbersome processes such as microdissection or macrodissection.

On the ddPCR chart, the *HER2* gene in tumor cells of a sample plotted on the line "$R = x+1$" is supposed to be amplified twice as much as *CEP17*. We confirmed this theory by performing a cell line assay. Furthermore, we

observed a certain level of measurement error in ddPCR. We calculated the 99% CI in the cell line assay and applied this CI as the "equivocal area" between positive and negative areas on the ddPCR–TCR chart with clinical cases. As a result, we identified all conventionally HER2-positive cases even when the ddPCR ratio was relatively low.

Three of the cases within this equivocal area were IHC score 3+ according to the ASCO guideline 2013 [7] (case numbers 32, 33, and 35 in Table 2), but these cases had been determined as IHC score 2+ (circumferential membrane staining of <30% in tumor cells) based on the ASCO guideline 2007 [8] at the time of diagnosis. One of them (case number 35) showed a relatively low ddPCR ratio because of heterogeneous expression of HER2 protein. The other two cases (32 and 33) had even been determined as "HER2 negative" by ISH. For these cases, the result of our method was very close to that of ISH rather than IHC. Conversely, three conventionally HER2-negative cases were within the equivocal area (case numbers 3, 4, and 8 in Table 2). The ddPCR ratio of cases 3 and 4 were relatively low. However, because of the low TCR, they were plotted in the equivocal area. Thus, the HER2 status of samples with a low TCR, such as <0.2, may not be accurately determined by our ddPCR–TCR method. Regarding case number 8, although it was determined as HER2 1+ by IHC, its ISH ratio was 1.86, which was determined as "equivocal" according to the ASCO/CAP guideline 2007 [8]. It may be quite difficult to completely remove such controversial cases with our range of the equivocal area.

By setting the equivocal area on the ddPCR–TCR chart, we can possibly identify such literally "equivocal" or "discrepant" cases without failure. Upon practical use of the ddPCR–TCR method, the decision of using the anti-HER2 antibody for cases included in the equivocal area should be made comprehensively, considering both the clinical and pathological information. In contrast, 21 out of 23 (91.3%) HER2 2+ cases, for which both IHC and subsequent ISH had been performed, were plotted on either "positive" or "negative" areas on the chart. This result indicates that using our method may dramatically reduce the need to perform ISH and thereby significantly reduce costs in determination of *HER2* gene amplification.

An important prerequisite for setting the cut-off line of "$x + 1$" in Fig. 1a is that gene copy numbers of cancer cells were homogeneous and that non-cancer cells were genetically stable with diploid chromosomes. We previously reported a study of determining *HER2* gene amplification in gastric cancer biopsies using dPCR, and developed a chart by the combination of the dPCR result and TCR [6]. On the chart for gastric cancer, the equivocal area was set by considering the high frequency of heterogeneity and CEP17 polysomy in gastric cancer. In breast cancer, heterogeneity is reported to be much less frequent than in gastric cancer [15]. In this study, three cases were heterogeneous for HER2 expression, which showed a mixture of a small proportion of focally well-stained spots and a large proportion of non-stained spots (case numbers 22, 31, and 35 in Table 2). In particular, case number 35 showed a relatively low ddPCR ratio considering its high ISH ratio. The equivocal area on the ddPCR–TCR chart was also helpful to identify such heterogeneous cases. However, CEP17 polysomy is reported to be often observed in breast cancer [16, 17]. We reviewed the sections subjected to DISH and searched for CEP17 polysomy based on a commonly adopted threshold, a mean of ≥3 CEP17 signals per nucleus [17]. As a result, there was no case of CEP17 polysomy but two HER2 3+ cases, numbers 37 and 38 in Table 2, in which CEP17 was amplified in association with highly amplified HER2, and thus high ddPCR ratio was not as high compared with their very high ISH ratios. In conclusion, we considered that tumor heterogeneity and CEP17 polysomy have little influence on our method for breast cancer. Interestingly, the ddPCR ratios of some cases were less than 1, especially case number five that showed a very low ratio (0.532). Reviewing the section of case number five used for DISH revealed that some tumor cells showed *HER2* gene monosomy or even complete deletion of *HER2*. Tubbs et al. [18] reported the frequency of *HER2* monoallelic deletion as 2% (12 of 742) in their assessment of the HER2 status in breast cancer by FISH. Because *HER2* gene deletion is a rare event, little is known about its clinical significance or the signaling pathway for tumor growth.

In this study, we tried to automate the process as much as possible. DNA extraction techniques used to be labor intensive and time consuming, but the MagCore® Genomic DNA FFPE One-Step Kit helped us to extract DNA from up to 16 samples in less than 3 h with only few manual procedures. The combination of MagCore® and ddPCR provides not only precise and objective quantification of the *HER2* gene copy number in breast cancer, but also decreases variation and error, reduces the time and labor of laboratory technicians and pathologists, and improves the cost-effectiveness of the overall method.

A reduction in the time required to determine the HER2 status can also help breast surgeons. Gagliato et al. [14] reported in their retrospective review that a delay in the initiation of adjuvant chemotherapy worsens survival outcomes, particularly in patients with trastuzumab-treated, HER2-positive breast tumors. With the use of ddPCR, surgeons will be able to initiate administration of anti-HER2 antibodies to patients with HER2-positive tumors at an earlier stage than what was previously possible.

We only analyzed surgical specimens from patients without a history of preceding chemotherapies. Further

studies are needed to assess biopsy specimens or samples from patients treated with neoadjuvant therapy.

In summary, we analyzed *HER2* gene amplification in FFPE tissue samples of 41 breast cancer cases by considering the ddPCR ratio and TCR. Our results showed high concordance with the conventional HER2 status. We succeeded in making most of the process automatic and obtained quite objective and reproducible data. Our findings do indicate that determining the HER2 status using the ddPCR–TCR method is definitely feasible in clinical practice as a tool to measure *HER2* gene amplification in FFPE samples of breast cancer patients. This method might complement or even replace conventional methods of examination in the future. A large-scale study is needed to examine whether the ddPCR–TCR method can be predictive and has prognostic value for the treatment of HER2-positive breast cancer patients with anti-HER2 antibodies.

Acknowledgements We are grateful to Aiko Nishimoto, Kei Sakuma, and Tami Ohmi for their technical support. We also thank Dr. Teppei Morikawa for statistical support.

Compliance with ethical standards

Conflict of interests No conflict of interests declared.

Ethical standards The study was carried out with permissions from the University of Tokyo Hospital Ethics Committee. Written informed consent was obtained from all participants.

References

1. Moelans CB, de Weger RA, Van der Wall E et al (2011) Current technologies for HER2 testing in breast cancer. Crit Rev Oncol/Hematol 80:380–392. doi:10.1016/j.critrevonc.2010.12.005
2. Vogelstein B, Kinzler KW (1999) Digital PCR. Proc Natl Acad Sci 96:9236–9241. doi:10.1073/pnas.96.16.9236
3. Whale AS, Huggett JF, Cowen S et al (2012) Comparison of microfluidic digital PCR and conventional quantitative PCR for measuring copy number variation. Nucleic Acids Res 40:e82. doi:10.1093/nar/gks203
4. Hindson BJ, Ness KD, Masquelier DA et al (2011) High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Anal Chem 83:8604–8610. doi:10.1021/ac202028g
5. Taylor SC, Carbonneau J, Shelton DN et al (2015) Optimization of droplet digital PCR from RNA and DNA extracts with direct comparison to RT-qPCR: clinical implications for quantification of Oseltamivir-resistant subpopulations. J Virol Methods 224:58–66. doi:10.1016/j.jviromet.2015.08.014
6. Matsusaka K, Ishikawa S, Nakayama A (2016) Tumor content chart-assisted HER2/CEP17 digital PCR analysis of gastric cancer biopsy specimens. PLoS ONE 11:e0154430. doi:10.1371/journal.pone.0154430
7. Wolff AC, Hammond ME, Hicks DG et al (2013) Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. J Clin Oncol 31:3997–4013. doi:10.1200/JCO.2013.50.9984
8. Wolff AC, Hammond ME, Schwartz JN et al (2007) American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Oncol 25:118–145. doi:10.1200/JCO.2006.09.2775
9. Gevensleben H, Garcia-Murillas I, Graeser MK et al (2013) Noninvasive detection of HER2 amplification with plasma DNA digital PCR. Clin Cancer Res 19:3276. doi:10.1158/1078-0432.ccr-12-3768
10. Garcia-Murillas I, Lambros M, Turner NC (2013) Determination of HER2 amplification status on tumour DNA by digital PCR. PLoS ONE 8:e83409. doi:10.1371/journal.pone.0083409
11. Braun M, Kirsten R, Rupp NJ et al (2013) Quantification of protein expression in cells and cellular subcompartments on immunohistochemical sections using a computer supported image analysis system. Histol Histopathol 28:605–610
12. Nikolaishvilli-Feinberg N, Cohen SM, Midkiff B et al (2013) Development of DNA damage response signaling biomarkers using automated, quantitative image analysis. J Histochem Cytochem 62:185–196. doi:10.1369/0022155413516469
13. Fisel P, Stühler V, Bedke J et al (2015) MCT4 surpasses the prognostic relevance of the ancillary protein CD147 in clear cell renal cell carcinoma. Oncotarget 6:30615–30627. doi:10.18632/oncotarget.5593
14. de Melo Gagliato D, Gonzalez-Angulo AM, Lei X et al (2014) Clinical impact of delaying initiation of adjuvant chemotherapy in patients with breast cancer. J Clin Oncol 32:735–744. doi:10.1200/JCO.2013.49.7693
15. Hofmann M, Stoss O, Shi D et al (2008) Assessment of a HER2 scoring system for gastric cancer: results from a validation study. Histopathology 52:797–805. doi:10.1111/j.1365-2559.2008.03028.x
16. Vranic S, Teruya B, Repertinger S et al (2010) Assessment of HER2 gene status in breast carcinomas with polysomy of chromosome 17. Cancer 117:48. doi:10.1002/cncr.25580
17. Hanna WM, Rüschoff J, Bilous M et al (2013) HER2 in situ hybridization in breast cancer: clinical implications of polysomy 17 and genetic heterogeneity. Mod Pathol 27:4. doi:10.1038/modpathol.2013.103
18. Tubbs RR, Hicks DG, Cook J et al (2007) Fluorescence in situ hybridization (FISH) as primary methodology for the assessment of HER2 Status in adenocarcinoma of the breast: a single institution experience. Diagn Mol Pathol 16:207–210. doi:10.1097/PDM.0b013e318064c72a