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Research Article

Droplet Digital PCR Using HER2/EIF2C1 Ratio for Detection of HER2 Amplification in FFPE Breast Cancer Tissues with Low or Equivocal HER2 Expression

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Abstract

Background: *HER2 amplification/overexpression is the predictive biomarker for HER2-targeted therapy. The aim of this study is to investigate whether droplet digital PCR (ddPCR) using the HER2/EIF2C1 ratio could be an alternative HER2 detection assay in formalin-fixed, paraffin-embedded (FFPE) BC tissues with low or equivocal HER2 expression.*

Methods and Patients: *We determined HER2 status by ddPCR in 150 FFPE BC tissues previously classified as IHC1+, IHC2+, and IHC3+; 90 of these were previously determined as FISH-negative and FISH-positive. Optimal cutoff thresholds for the HER2/EIF2C1 ratio, determined by the receiver operating characteristics (ROC) curve, were 2.72 (98% sensitivity, 88% specificity) and 2.64 (89.23% sensitivity, 92% specificity) using IHC and FISH as standard methods, respectively.*

Results: *The concordance rate of HER2 status (n=144) determined by IHC/FISH and ddPCR was 89.58% (kappa=0.791, 89.85% sensitivity, 89.33% specificity). The HER2/EIF2C1 ratio in the IHC3+ group was significantly higher than in IHC1+ and IHC2+ groups (P<0.0001). In IHC3+, the concordance between IHC/FISH and ddPCR was 98% (kappa=1.00). In IHC2+ (n=44), the concordance between FISH and ddPCR was 79.54% (kappa=0.579, 65% sensitivity, 91.7% specificity); the HER2/EIF2C1 ratio in FISH-positive cases was significantly higher than in FISH-negative cases (P<0.001). Interestingly, 12% of IHC1+ cases showed HER2 amplification by ddPCR.*

Conclusion: *Thus, ddPCR using the HER2/EIF2C1 ratio is a robust, sensitive, and accurate assay and represents an alternative method to determine HER2 amplification. This technique should be used to clarify HER2 amplification in breast cancer patients with low or equivocal HER2 expression (IHC1+ or IHC2+ with FISH-negative), which may benefit from novel HER2-directed ADCs. The heterogeneity of HER2-expressing cells contributes to discordant results between ddPCR and FISH.*

Keywords: ddPCR, EIF2C1, FFPE, FISH, HER2, IHC

Abbreviations

ADC: Antibody-Drug Conjugate
BC: Breast Cancer
CEP17: Chromosome 17 Centromere
ddPCR: Droplet Digital PCR
EIF2C1: Human Eukaryotic Translation Initiation Factor 2C1
FFPE Tissues: Formalin-Fixed Paraffin-Embedded Tissues
FISH: Fluorescence In Situ Hybridization
HER2: Human Epidermal Growth Factor Receptor 2
IHC: Immunohistochemistry

1. Introduction

Amplification and overexpression of the HER2/Neu gene are detected in approximately 25%–30% of breast cancers (BC) and are strongly associated with poor prognosis [1, 2]. HER2 status has a therapeutic impact because monoclonal antibodies against HER2 (e.g., trastuzumab and pertuzumab) combined with chemotherapy, as well as HER2-directed antibody-drug conjugate (trastuzumab emtansine), are effective for treating patients with HER2-positive BC [3-6]. According to the testing algorithm recommended by the American Society of Clinical Oncology (ASCO)/ College of American Pathologists (CAP), immunohistochemistry (IHC) to detect HER2 protein overexpression is performed first in many laboratories; fluorescence in situ hybridization (FISH) to detect HER2 gene amplification is required if the results of HER2 IHC are equivocal, i.e., IHC2+ [7, 8]. However, both methods have technical limitations; HER2 FISH is costly and time-consuming, whereas HER2 IHC interpretation could be subjective and variable among evaluators and antibodies.

Based on recent studies, droplet digital PCR (ddPCR) has been proposed as a novel method to quantitate HER2 gene copy number variation in DNA extracted from formalin-fixed, paraffin-embedded (FFPE) BC tissues [9-13]. Our previous study has also shown that HER2 status determined by ddPCR using the HER2/EIF2C1 ratio in frozen BC tissues has high sensitivity (90%), high specificity (85.7%), and high concordance (87.7%) with HER2 status determined by HER2 FISH using HER2/CEP17 ratio [14]. However, the number of frozen tissues in our previous study was limited. Furthermore, novel HER2-directed ADCs (trastuzumab deruxtecan, trastuzumab duocarmazine, and disitamab vedotin) have recently shown benefits in patients with HER2-negative BC with low HER2 expression (i.e., IHC1+ or IHC2+ with FISH-negative) [15-17].

Therefore, this study investigated whether ddPCR using the HER2/EIF2C1 ratio could be an alternative HER2 detection assay in FFPE BC tissues with low or equivocal HER2 expression. We determined HER2 status by ddPCR using the HER2/EIF2C1 ratio in 150 FFPE BC tissues previously scored by HER2 IHC as IHC1+ (n=50), IHC2+ (n=50), and IHC3+ (n=50); 90 of these tissues were previously determined as HER2-non-amplified (FISH-negative, n=25), and HER2-amplified (FISH-positive, n=65). The optimal cutoff thresholds for the HER2/EIF2C1 ratio were determined by the receiver operating characteristics (ROC) curve using both HER2 IHC and HER2 FISH as standard methods. The concordance between ddPCR and IHC/FISH was investigated. In addition, the sensitivity and specificity of HER2 status determined by ddPCR in FFPE BC tissues were evaluated.

2. Materials and Methods

2.1 DNA Extraction

FFPE BC tissues were obtained from the Department of Pathology at Ramathibodi Hospital. One hundred fifty FFPE BC tissues, in which HER2 expression was previously scored as IHC1+, IHC2+, and IHC3+, were selected by an experienced breast pathologist. Five serial 4-µm sections of individual tissue were used for DNA extraction using QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. The DNA quantity was measured by NanoDrop 2000 (Thermo Scientific, USA).

2.2 Droplet Digital PCR (ddPCR)

Droplet digital PCR was performed using the ddPCR method on a Bio-Rad QX200™ (Bio-Rad, Hercules, CA, USA) following our previous report [14]. Briefly, a total PCR reaction of 20 µl was prepared with 15–20 ng DNA and 2× ddPCR Supermix for the probe (BioRad, Hercules, CA, USA); primers and fluorescent probes (FAM and VIC) were prepared from the Prime PCR assay for ddPCR (dHsaCP1000116 for HER2 and dHsaCP2500349 for EIF2C1 as the reference control). The total 40-µl emulsified PCR reaction volumes were transferred to a 96-well plate and heat-sealed before running on a T1000 thermal cycler (Bio-Rad, Hercules, CA, USA) using the following cycle: 95°C for 10 min, 40 cycles of 94°C for 30 s and 60°C for 60 s, followed by 98°C for 10 min, and hold at 4°C. The temperature ramp rate was 2°C/s for all steps. Positive and negative controls were included in each run. After the PCR, the plates were transferred to a Bio-Rad QX200 droplet reader. Analysis of ddPCR data was performed using QuantaSoft v1.3.2.0 software from Bio-Rad. The ROC curve analysis defined the optimal cutoff values of the HER2/EIF2C1 ratio (>2.72 and >2.64) as HER2 amplification.

2.3 Fluorescence In Situ Hybridization (FISH)

Standard FISH assays to detect HER2 gene amplification (HER2/CEP17 > 2) were performed in the Human Genetic Laboratory, Department of Pathology, following the ASCO/CAP guideline [7, 8]. HER2 status determined by FISH and the patients' clinicopathological characteristics were obtained by reviewing the electronic medical records of Ramathibodi Hospital.

2.4 Immunohistochemistry (IHC)

HER2 protein overexpression was determined by IHC, according to the standard method as previously described, and scored following the ASCO/CAP guidelines as negative (IHC 0, 1+), equivocal (IHC2+), or positive (IHC3+) [7, 8, 18].

2.5 Statistical analysis

Statistical analyses were performed using SPSS v.11.5 (SPSS Inc., Chicago, Illinois, USA) or GraphPad Prism 7 (version 7.03). Mann–Whitney U test was applied to determine significant differences between groups ($P<0.01$). The optimal cutoff value was determined by the ROC curve. The Kappa coefficient was used to determine the concordance between IHC, FISH, and ddPCR methods in determining HER2 status. A kappa coefficient of <0.2 was considered poor, 0.21–0.40 fair, 0.41–0.6 moderate, 0.61–0.8 substantial, and 0.81–1 almost perfect agreement.

3. Results

One hundred fifty FFPE BC tissues, in which IHC previously scored HER2 expression as IHC1+ (n=50), IHC2+ (n=50), and IHC3+ (n=50), were selected to determine HER2 status by ddPCR using EIF2C1 as the reference gene. The clinicopathological characteristics of the 150 patients are shown in Table 1.

Characteristics	Number of patients	%
Age (years)		
< 50	42	28
≥ 50	108	72
Range 30-92, Median 57		
Tumor stage		
T1	46	30.67
T2	72	48
T3	11	7.33
ND	21	14
Tumor grade		
1-2	72	48
3	59	39.33
ND	19	12.67
Lymph node status		
Negative	76	50.46
Positive	52	34.67
ND	22	14.67
Estrogen receptor		
Negative	45	30
Positive	104	69.3
ND	1	0.7
Progesterone receptor		
Negative	59	39.3
Positive	91	60.7
Ki-67		
< 10%	7	4.7
≥ 10%	143	95.3
HER2 status (IHC)		
1+	50	33.33
2+	50	33.33
3+	50	33.33
HER2 status (FISH)		
Negative	25	27.78
Positive	65	72.22
ND, no data available		

Table 1: Clinicopathological characteristics of patients with invasive breast carcinomas (n=150)

We determined the optimal cutoff value of the HER2/EIF2C1 ratio detected by ddPCR using HER2 IHC as the standard reference method; DNA from FFPE BC tissues with HER2 IHC1+ (n=50) and IHC3+ (n=50) was defined as HER2 IHC-negative and IHC-positive, respectively. The ddPCR results were analyzed by the ROC curve. The area under the curve (AUC) of the ddPCR HER2/EIF2C1 ratio was 0.9772 [95% Confidence Interval (CI) 0.9455–1.009, P<0.0001)]. The optimal cutoff ratio (>2.72) was identified. The sensitivity and specificity were 98% and 88%, respectively, as shown in Fig. 1a and 1c and Table 2.

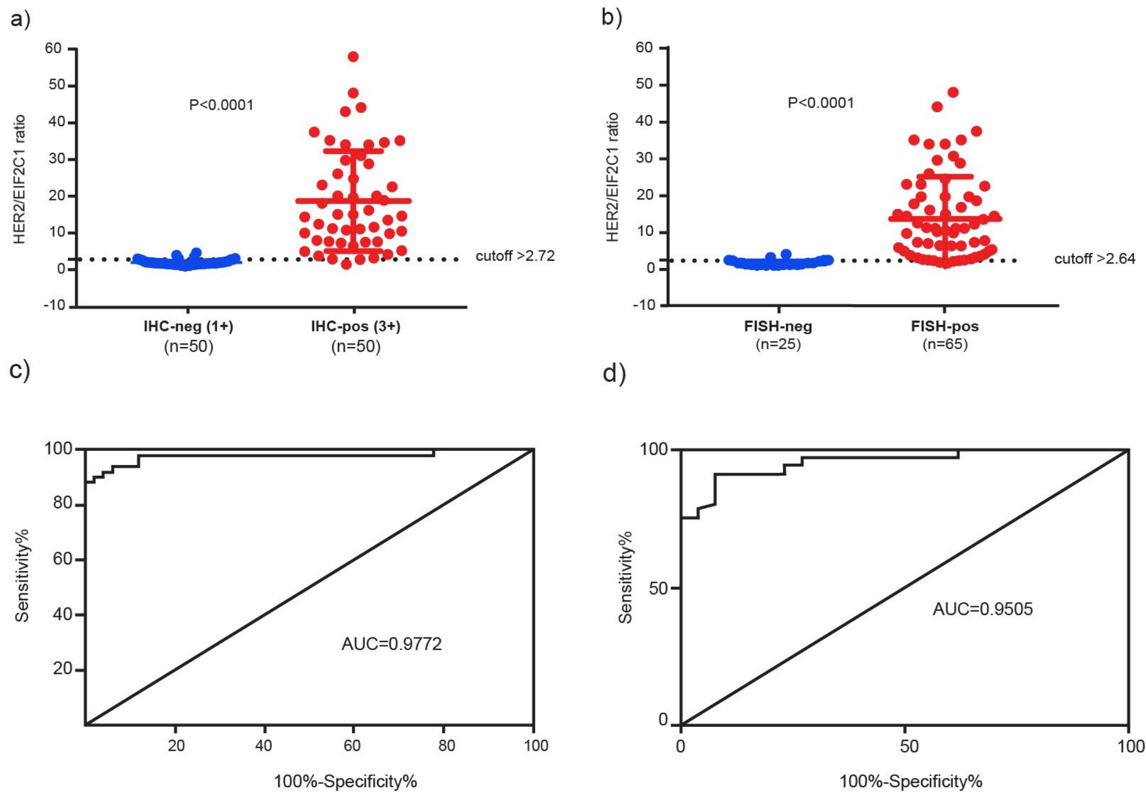

Figure 1: The HER2/EIF2C1 ratio determined by ddPCR in FFPE BC tissues and the ROC curve analysis
HER2/EIF2C1 ratio was assessed by ddPCR in DNA from tissues previously determined by HER2 IHC, (a), or HER2 FISH, (b). Each dot represents the mean value of the HER2/EIF2C1 ratio for an individual sample, and the mean ± SD error bar for the groups is shown (P<0.0001). ROC curve, as shown in (c) and (d), determined the optimal cutoff ratio based on the ddPCR results from (a) (the cutoff HER2/EIF2C1 ratio >2.72 with 98% sensitivity, 88% specificity, AUC=0.9772) and (b) (the cutoff HER2/EIF2C1 ratio >2.64 with 89.23% sensitivity, 92% specificity, AUC=0.9505), respectively.

IHC Test	Score	N (%)	HER2/EIF2C1 ratio	HER2/EIF2C1 ratio ≤ 2.72	HER2/EIF2C1 ratio > 2.72	Cutoff	AUC	Sensitivity	Specificity
			Median (range)	N (%)	N (%)			(%)	(%)
IHC-negative	1+	50 (100)	1.68 (0.84–4.5)	44 (88)	6 (12)	2.72	0.9772	98	88
IHC-positive	3+	50 (100)	1.47 (1.43–58)	1 (2)	49 (98)				
FISH Test	HER2/CEP17 ratio	N (%)	HER2/EIF2C1 ratio	HER2/EIF2C1 ratio ≤ 2.64	HER2/EIF2C1 ratio > 2.64	Cutoff	AUC	Sensitivity	Specificity
			Median (range)	N (%)	N (%)			(%)	(%)
FISH-negative	≤ 2	25 (100)	1.73 (1–4.22)	23 (92)	2 (8)	2.64	0.9505	89.23	92
FISH-positive	> 2	65 (100)	10.85 (1.67–58)	7 (10.77)	58 (89.23)				

Table 2: Cutoff values for HER2 amplification determined by ddPCR and ROC using IHC and FISH as standard methods.

In addition, we also determined the optimal cutoff value of the HER2/EIF2C1 ratio detected by ddPCR using HER2 FISH as the standard reference method; among 150 tissues, only 90 tissues were previously determined as HER2-non-amplified (FISH-negative, n=25) and HER2-amplified (FISH-positive, n=65). The ROC curve analyzed the ddPCR results using HER2 FISH as the reference standard method. The AUC of the ddPCR HER2/EIF2C1 ratio was 0.9505 (95% CI 0.9097–0.9912, P<0.0001). The optimal cutoff ratio (>2.64) was identified. The sensitivity and specificity were 89.23% and 92%, respectively, as shown in Fig. 1b and 1d and Table 2.

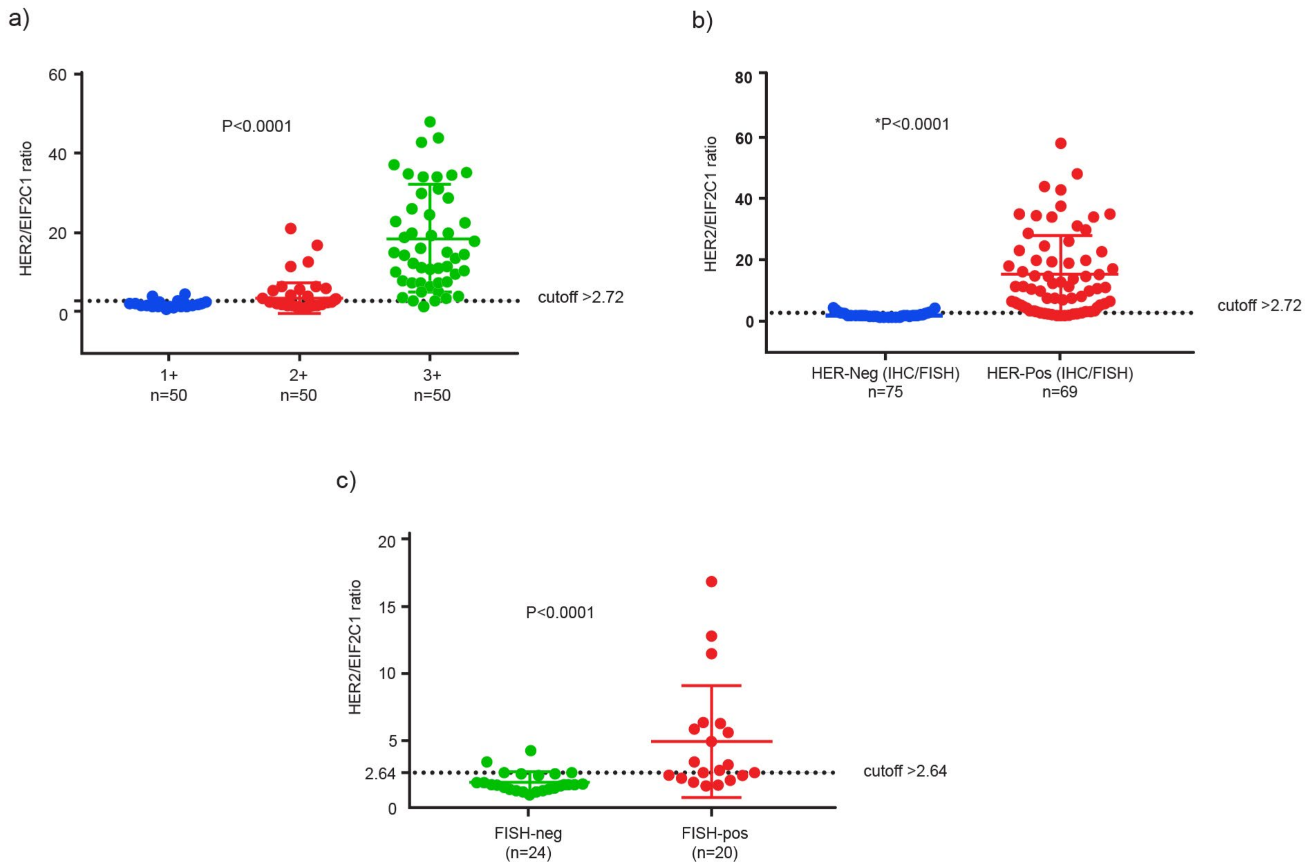

Figure 2: Comparison of HER2/EIF2C1 ratio from ddPCR assay in various groups of FFPF BC tissues
(a) HER2/EIF2C1 ratio was assessed by ddPCR among 150 tissues previously determined by HER2 IHC as 1+, 2+, and 3+, respectively. (b) 144 tissues previously determined by IHC/FISH as HER2-negative and HER2-positively. (c) HER2 amplification was assessed by FISH and ddPCR among 44 tissues previously determined by HER2 IHC as IHC2+. Each dot represents the mean value of the HER2/EIF2C1 ratio for an individual sample, and the mean ± SD error bar for the groups is shown (p<0.0001).

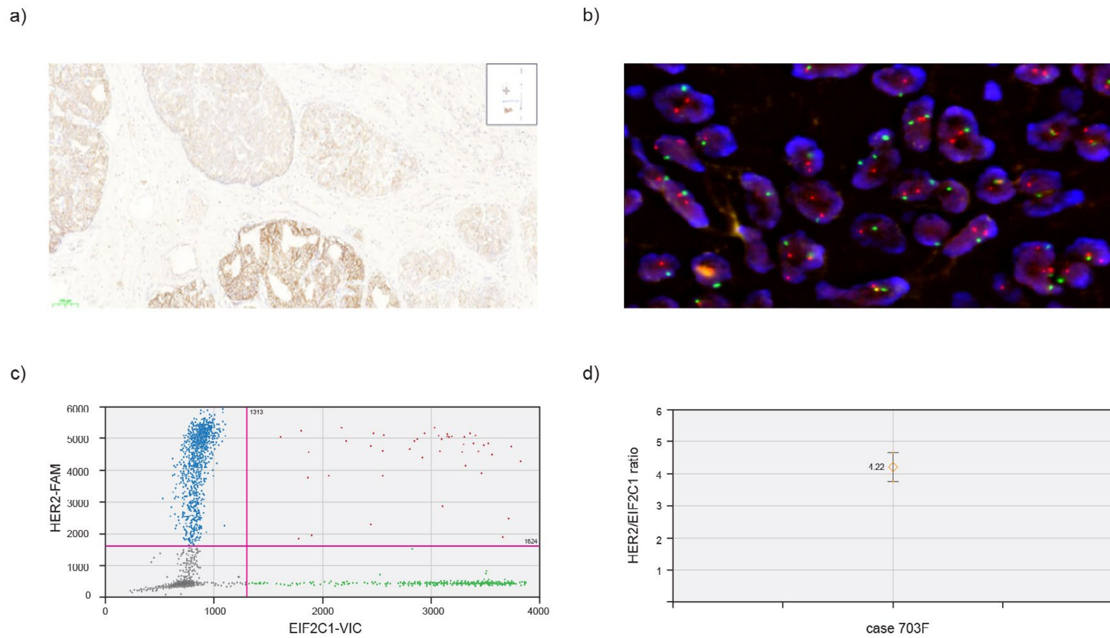

Figure 3: Representative of FFPE BC tissue (case 703F) with HER2 IHC2+/FISH-negative/ddPCR-positive
(a) HER2 IHC2+ (10X), (b) HER2 FISH-negative (HER2/CEP17 ratio=1.32). PathVysion HER2 FISH assay using fluorophore-labeled HER2 (orange signals) and fluorophore-labeled chromosome 17 centromere (green signals). (c) HER2 ddPCR-positive shows signals of HER2-FAM vs. EIF2C1-VIC. HER2 and EIF2C1 were labeled with FAM and VIC fluorescent probes, respectively. The four quadrants represent the top left: droplets with HER2 DNA only; top right: droplets with both HER2 and EIF2C1 DNA; bottom right: droplets with EIF2C1 DNA only; and bottom left: droplets with no DNA. (d) HER2 ddPCR-positive shows HER2/EIF2C1 ratio of 4.22 in the tissue.

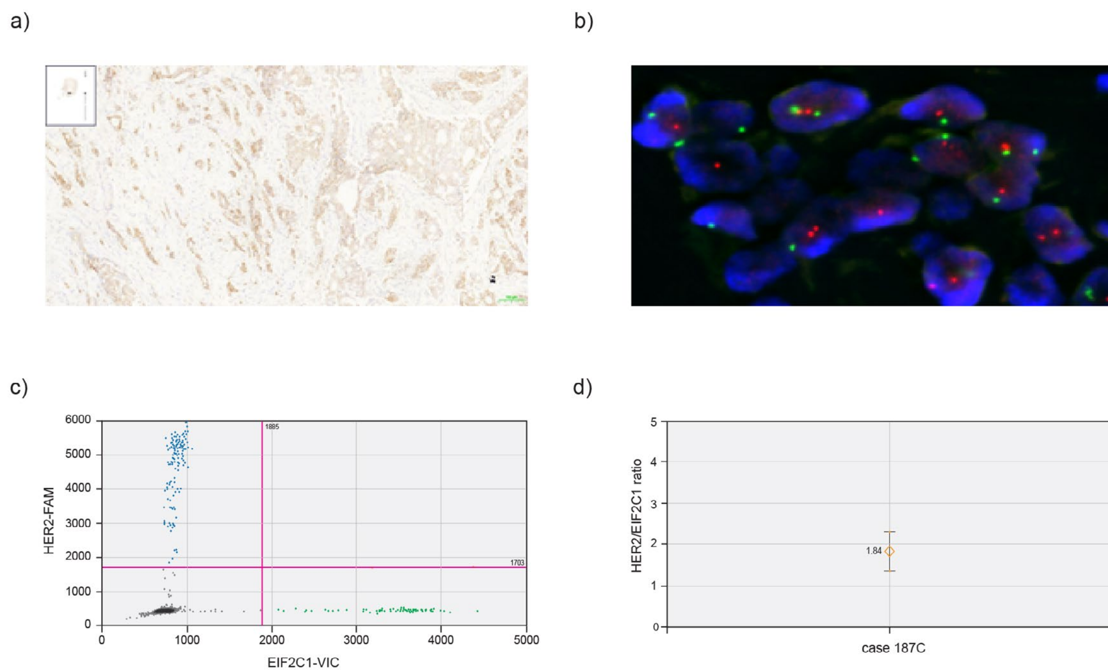

Figure 4: Representative of FFPE BC tissue (case 187C) with HER2 IHC2+/FISH-negative/ddPCR-negative
(a) HER2 IHC2+ (10X), (b) HER2 FISH-negative (HER2/CEP17 ratio=1.26). PathVysion HER2 FISH assay using fluorophore-labeled HER2 (orange signals) and fluorophore-labeled chromosome 17 centromere (green signals). (c) HER2 ddPCR-negative shows signals of HER2-FAM vs. EIF2C1-VIC. HER2 and EIF2C1 were labeled with FAM and VIC fluorescent probes, respectively. The four quadrants represent the top left: droplets with HER2 DNA only; top right: droplets with both HER2 and EIF2C1 DNA; bottom right: droplets with EIF2C1 DNA only; and bottom left: droplets with no DNA. (d) HER2 ddPCR-negative shows a HER2/EIF2C1 ratio of 1.84 in the tissue.

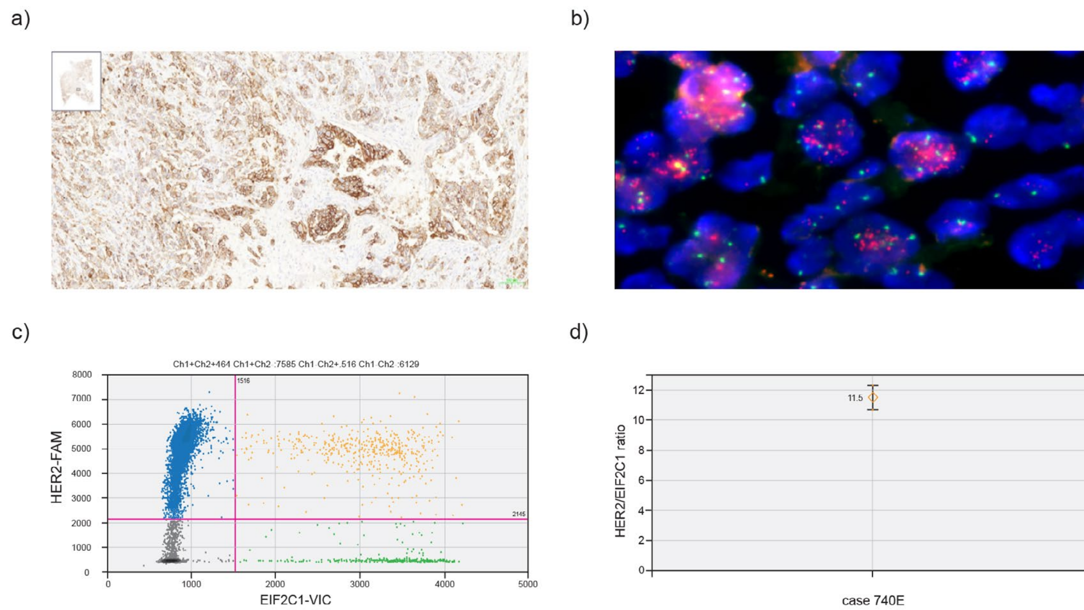

Figure 5: Representative of FFPE BC tissue (case 740E) with HER2 IHC2+/FISH-positive/ddPCR-positive
(a) HER2 IHC2+ (10x), (b) HER2 FISH-positive (HER2/CEP17 ratio=6.95). PathVysion HER2 FISH assay using fluorophore-labeled HER2 (orange signals) and fluorophore-labeled chromosome 17 centromere (green signals). (c) HER2 ddPCR-positive shows signals of HER2-FAM vs. EIF2C1-VIC. HER2 and EIF2C1 were labeled with FAM and VIC fluorescent probes, respectively. The four quadrants represent the top left: droplets with HER2 DNA only; top right: droplets with both HER2 and EIF2C1 DNA; bottom right: droplets with EIF2C1 DNA only; and bottom left: droplets with no DNA. (d) HER2 ddPCR-positive shows HER2/EIF2C1 ratio of 11.5 in the tissue.

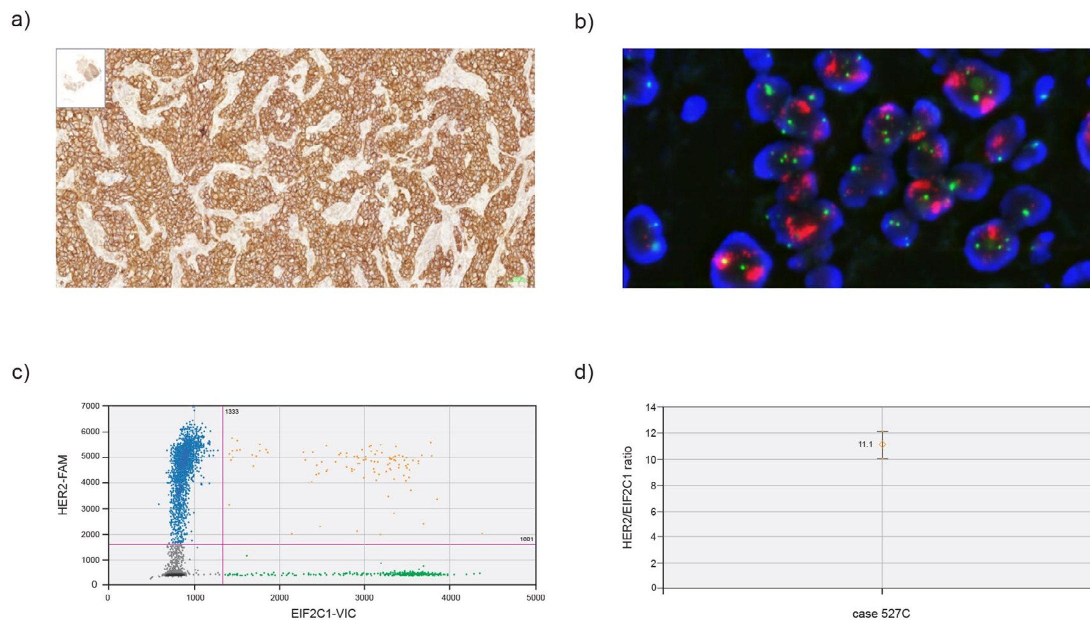

Figure 6: Representative of FFPE BC tissue (case 527C) with HER2 IHC3+/FISH-positive/ddPCR-positive
(a) HER2 IHC3+ (10X), (b) HER2 FISH-positive (HER2/CEP17 ratio=10.09). PathVysion HER2 FISH assay using fluorophore-labeled HER2 (orange signals) and fluorophore-labeled chromosome 17 centromere (green signals). (c) HER2 ddPCR-positive shows signals of HER2-FAM vs. EIF2C1-VIC. HER2 and EIF2C1 were labeled with FAM and VIC fluorescent probes, respectively. The four quadrants represent the top left: droplets with HER2 DNA only; top right: droplets with both HER2 and EIF2C1 DNA; bottom right: droplets with EIF2C1 DNA only; and bottom left: droplets with no DNA. (d) HER2 ddPCR-positive shows HER2/EIF2C1 ratio of 11.1 in the tissue.

		ddPCR (HER2/EIF2C1)		Kappa coefficient
		Negative (ratio ≤ 2.72)	Positive (ratio > 2.72)	0.791
IHC/FISH (n=144)	Negative (IHC1+ and IHC2+/FISH-negative)	67 (89.33%)	8 (10.67%)	
	(IHC3+ and IHC2+/FISH-positive)	7 (10.15%)	62 (89.85%)	
		ddPCR (HER2/EIF2C1)		Kappa coefficient
		Negative (ratio ≤ 2.64)	Positive (ratio > 2.64)	0.579
FISH (IHC2+, n=44)	Negative (HER2/CEP17 ratio ≤ 2)	22 (91.7%)	2 (8.3%)	
	Positive (HER2/CEP17 ratio > 2)	7 (35%)	13 (65%)	

Table 3: HER2 status in FFPE breast cancer tissues determined by ddPCR using cutoff 2.72 compared with IHC/FISH (n=144), and using cutoff 2.64 compared with FISH in FFPE tissues with HER2 IHC2+ (n=44)

IHC score	FISH (HER2/CEP17)		ddPCR (HER2/EIF2C1)		Kappa coefficient
	ratio <2	ratio ≥2	ratio ≤2.72	ratio >2.72	
1+ (n=50)	-	-	44/50 (88%)	6/50 (12%)	-
2+ (n=50)	24/44 (54.6%)	20/44 (45.4%)	35/50 (70%)	15/50 (30%)	0.579
3+ (n=50)	1/46 (2.2%)	45/46 (97.8)	1/50 (2%)	49/50 (98%)	1.00

Table 4: Comparison of HER2 status determined by IHC, FISH, and ddPCR

Of the 150 FFPE BC tissues, the median HER2/EIF2C1 ratios in tissues with HER2 IHC1+ (n=50), IHC2+ (n=50), and IHC3+ (n=50) were 1.68, 2.31, and 14.4, respectively. The HER2/EIF2C1 ratio in the IHC3+ group was significantly higher than in IHC1+ and IHC2+ groups (P<0.0001), as shown in Fig. 2a. The HER2/EIF2C1 ratio in HER2-positive group by IHC/FISH, i.e., IHC3+ and IHC2+/FISH-positive, was significantly higher than in HER2-negative group by IHC/FISH, i.e., IHC1+ and IHC2+/FISH-negative, (P<0.0001) as shown in Fig. 2b. Among the 144 FFPE BC tissues, the concordance rate of HER2 status determined by IHC/FISH and ddPCR was 89.58% (kappa=0.791) with 89.85% sensitivity and 89.33% specificity (Table 3). Furthermore, in the IHC2+ group (n=44), the median HER2/EIF2C1 ratio in FISH-positive cases (3.04; range 1.67-16.9) was significantly higher than in FISH-negative cases (1.73; range 1-4.22, P<0.001) as shown in Fig. 2c, and the concordance rate of HER2 status determined by FISH and ddPCR (n=44) was 79.54% (kappa=0.579) with 65% sensitivity and 91.7% specificity (Table 3). In the IHC1+ group, 12% (6/50) of FFPE BC tissues (cases 960F, 150H, 151F, 225K, 325A, and 239D) were classified as HER2-positive (HER2-amplified, HER2/EIF2C1 ratio >2.72) by ddPCR (Table 4). Of the IHC2+ group (n=50), we found HER2-positive in 45.45% (20/44) by FISH and 30% (15/50) by ddPCR. Two cases (8.3%, cases 703F and 203B) with discordant results in the IHC2+/FISH-negative group were classified as ddPCR-positive (HER2- amplified) by ddPCR. Representative IHC2+/FISH-negative/ddPCR-positive FFPE BC tissue (case 703F) is shown in Fig. 3. In contrast, representative IHC2+/FISH-negative/ddPCR-negative FFPE BC tissue (case 187C) is shown in Fig. 4. In the IHC2+/FISH-positive cases, the concordant result was 65% (13/20); the discordant result was 35% (7/20) by ddPCR (cases 734B, 063J, 784B, 687E, 951C, 687I, and 231D). Representative IHC2+/FISH-positive/ddPCR-positive FFPE BC tissue (case 740E) is shown in Fig. 5. Forty-six IHC3+ samples were determined by FISH and classified as HER2 FISH-negative in 2.2% (1/46, case 422E) and FISH-positive in 97.8% (45/46). The median HER2/EIF2C1 ratio in IHC3+/FISH-positive cases (14.5; range 2.74-58) was significantly higher than in the IHC3+/FISH-negative case (1.43; P<0.001). The concordance rate of HER2 status determined by FISH and ddPCR in IHC 3+ samples was 100% (kappa =1.00). Representative IHC3+/FISH-positive/ddPCR-positive FFPE BC tissue (case 527C) is shown in Fig. 6.

4. Discussion

Droplet digital PCR (ddPCR) is a high-throughput, high-precision, and highly sensitive technique for nucleic acid quantification. Previous studies on HER2 amplification detection in breast and gastric cancers have shown good concordance among ddPCR, IHC, and FISH assays [9-14, 19, 20]. However, studies of HER2 amplification detected by ddPCR in tissues with equivocal HER2 expression (i.e., IHC2+) are limited [11, 13].

In this study, HER2 amplification was determined in 150 FFPE BC tissues by ddPCR using the HER2/EIF2C1 ratio. Two cutoff thresholds (2.72 and 2.64) were obtained using IHC and FISH as standard methods, respectively. Of 144 breast cancer tissues tested, the concordance rate of HER2 status determined by IHC/FISH and ddPCR was substantial (kappa=0.791), with high sensitivity and specificity. However, we found that 12% of the tissues with HER2

IHC1+ showed HER2 amplification, as detected by ddPCR. Similarly, Owens et al. reported that 7.4% of tissues with HER2 IHC1+ showed HER2 amplification, as detected by FISH [21]. Before the arrival of novel HER2-directed ADCs, patients with HER2 IHC1+ were usually not further tested and not selected for HER2-targeted therapy. HER2 equivocal (IHC2+) results have been reported in 24% of BCs, and 23.3% of the tissues with HER2 IHC2+ showed HER2 amplification by FISH analysis [21, 22]. Furthermore, in this study, HER2 amplification was detected in the HER2 IHC2+ group at rates of 45.5% by FISH and 34% by ddPCR. Detection of HER2 amplification by ddPCR in cases with HER2 equivocal (IHC2+) expression was reported by Wang et al.; they used CEP17 as the reference control with a cutoff threshold of 1.62, yielding a sensitivity and specificity of 79.3% and 97.0%, respectively [13]. In contrast, we used EIF2C1 as the reference control to avoid co-amplification of HER2 and CEP17, as in our previous study [14]. Wang et al. reported that, in HER2 equivocal (IHC2+) cases, 75% of those with HER2 amplification by FISH were classified as HER2-positive by ddPCR, and 95% of those with HER2 non-amplification by FISH were classified as HER2-negative by ddPCR, with kappa=0.709 [13]. Consistently, we found in the present study that the concordance rate of HER2 status determined by FISH and ddPCR was 65% in the HER2 IHC2+/FISH-positive group and 91.7% in the HER2 IHC2+/FISH-negative group. Two of 24 FISH-negative cases (8.3%) (cases 703F and 023B) were determined as HER2-positive by ddPCR. These discordant results might be due to the heterogeneity of HER2 expression in the tumor cells, affecting the FISH assay in that HER2 signals might be counted in the HER2-non-amplified areas of the FFPE section, as shown in Fig. 3 (case703F). In HER2 equivocal (IHC 2+) cases (n=44), 35% of those determined to be HER2-positive by FISH were identified as HER2-negative by ddPCR, i.e., the HER2/EIF2C1 ratio < 2.64. In HER2 equivocal (IHC 2+) cases, the cutoff threshold of 2.64 was more sensitive than the cutoff threshold of 2.72 (65% vs. 55%), with the same specificity (91.7%).

In addition, the concordance rate in the HER2 IHC3+/FISH-positive group was 98% (kappa=1.00). The one discordant case was found to be HER2-negative by both ddPCR (HER2/EIF2C1 ratio=1.43) and FISH (HER2/CEP17 ratio=1.88). However, we found later that the pathologist had reviewed and reclassified this tissue as HER2 IHC2+. These results suggested that HER2 status categorized by IHC as IHC3+ had 100% concordance with ddPCR. Of note, the concordance between ddPCR and standard IHC or FISH methods was dependent on the HER2 status of the tissues. The most discordant cases were those with HER2 IHC2+/FISH-positive. These discordant results might arise from the heterogeneity of HER2-expressing cells due to either the distribution (clustered vs. dispersed) or the intensity of HER2 expression.

In conclusion, ddPCR using the HER2/EIF2C1 ratio is a robust, sensitive, and accurate assay and represents an alternative method to determine HER2 amplification. This technique should be used to clarify HER2 amplification in breast cancer patients with low or equivocal HER2 expression (IHC1+ or IHC2+ with FISH-negative), which may benefit from novel HER2-directed ADCs. The heterogeneity of HER2-expressing cells contributes to discordant results between ddPCR and FISH.

Statements & Declarations

Acknowledgments

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Competing Interests

The authors declare no conflict of interest regarding the publication of this paper.

Author Contributions

Anchalee Tantiwetrueangdet and Ravat Panvichian contributed to the study's conception and design. Anchalee Tantiwetrueangdet, Sansanee Wongwaisayawan, Pitichai Phornsarayuth, Ravat Panvichian, and Panuwat Lertsithichai performed material preparation, data collection, and analysis. The first draft of the manuscript was written by Anchalee Tantiwetrueangdet and edited by Ravat Panvichian, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

All data generated or analyzed during this study are included in this published article.

Ethics Approval

This study was approved by the Ethics Committee on Research involving Human Subjects of the Faculty of Medicine, Ramathibodi Hospital, Mahidol University.

Informed Consent

Because this retrospective study was observational and linked anonymized, no patient informed consent was required.

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