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Brief Communication

Droplet Digital PCR Measurement of *HER2* Copy Number Alteration in Formalin-Fixed Paraffin-Embedded Breast Carcinoma Tissue

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BACKGROUND: Human epidermal growth factor receptor 2 (HER2) testing is routinely performed by immunohistochemistry (IHC) and/or fluorescence in situ hybridization (FISH) analyses for all new cases of invasive breast carcinoma. IHC is easier to perform, but analysis can be subjective and variable. FISH offers better diagnostic accuracy and added confidence, particularly when it is used to supplement weak IHC signals, but it is more labor intensive and costly than IHC. We examined the performance of droplet digital PCR (ddPCR) as a more precise and less subjective alternative for quantifying *HER2* DNA amplification.

METHODS: Thirty-nine cases of invasive breast carcinoma containing ≥30% tumor were classified as positive or negative for HER2 by IHC, FISH, or both. DNA for these cases was extracted from formalin-fixed paraffin-embedded (FFPE) tissue for estimating the molecular copy number by ddPCR. ddPCR involved emulsifying hydrolysis probe–based PCR reaction mixtures containing the *ERBB2* [v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian); also known as *HER2*] gene and chromosome 17 centromere assays into nanoliter-sized droplets for thermal cycling and analysis.

RESULTS: ddPCR distinguished, through differences in the level of *HER2* amplification, the 10 *HER2*-positive samples from the 29 *HER2*-negative samples with 100% concordance of with HER2 status obtained by FISH and IHC analysis. ddPCR results agreed with the FISH results for the 6 cases that were equivocal in IHC analyses, confirming 2 of these samples as positive for *HER2* and the other 4 as negative.

CONCLUSIONS: ddPCR can be used as a molecular-analysis tool to precisely measure copy number alterations in FFPE samples of heterogeneous breast tumor tissue.

In 2011, >230 000 women were diagnosed with breast cancer in the US *(1)*. About 15%–25% of these cases show amplification of the *ERBB2* gene [v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian); also known as *HER2* (human epidermal growth factor receptor 2)] *(2)*. Excessive concentrations of the HER2 protein are associated with a more aggressive clinical course. Patients with high concentrations of HER2 protein are eligible for treatment with trastuzumab (Herceptin) *(3)*, a monoclonal antibody–based therapy. The standard 1-year course of trastuzamab is costly, and the drug can cause serious cardiac side effects, although they are rare *(4)*. Therefore, accurate assessment of HER2 status is critical for predicting prognosis and determining whether tailored therapeutics may be effective.

Clinical-testing guidelines recommended by the American Society of Clinical Oncology (ASCO)[3] and the College of American Pathologists (CAP) to increase the diagnostic accuracy of assessing HER2 status include using a combination of immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) testing *(5)*. Both methods can be routinely performed on formalin-fixed paraffin-embedded (FFPE) tissue sections. IHC scoring (0, 1+, 2+, 3+) is based on the relative intensity of tumor cell membrane staining with the HER2 antibody, whereas FISH scoring is based on counting the signals corresponding to immobilized fluorescent HER2 and chromosome 17 centromere (CEP17) hybridization probes. Most laboratories find HER2 testing with IHC to be easier to perform, but analysis of the results can be subjective and be variable with different antibodies and observers. FISH offers better diagnostic accuracy and added confidence, particularly when it is used to supplement weak IHC signals, but it is more labor intensive, time-consuming (e.g., the Dako HER2 FISH pharmDx™ test requires >14 h to complete), and costly than IHC.

To achieve a higher throughput capability and a more accurate molecular diagnosis, laboratories have been evaluating *HER2* copy number quantification

[3] Nonstandard abbreviations: ASCO, American Society of Clinical Oncology; CAP, College of American Pathologists; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization; FFPE, formalin-fixed paraffin-embedded; CEP17, chromosome 17 centromere; ddPCR, droplet digital PCR.

with real-time or quantitative PCR with external calibrators *(6)* or internal competitor calibrators *(7)*. Both approaches are relative-quantification strategies, but reports of limitations in precision have prevented distinguishing between small differences in copy number among samples, particularly with heterogeneous samples *(8)*.

The inherent quantification constraints of "analog" quantitative PCR have drawn investigators toward digital PCR to attain analytical results with lower imprecision *(9–16)*. Digital PCR is practiced most effectively by partitioning the PCR reaction mixture into thousands of compartments so that each compartment contains either 1 or 0 molecules of target DNA or RNA. The partitions then undergo thermal cycling to generate an amplified end product. A positive fluorescence signal is present only in the compartments that contained a target molecule. Precise, absolute quantification of the number of target DNA molecules in the reaction is simply achieved by counting the number of positive and negative compartments. The various modalities of compartmentalization that have been described for performing digital PCR include microtiter plates *(9)*, microwells *(14)*, microchambers *(12, 13)*, and droplets *(15, 16)*. The approach selected for this study was droplet digital PCR (ddPCR), because it offers a high level of partitioning at a low cost compared with other fixed-hardware configurations *(17)*.

In a previous study *(18)*, we demonstrated that ddPCR could be used to assess *HER2* transcript levels in FFPE samples of human breast tumors. For the present work, we describe our results showing that ddPCR can also measure *HER2* copy number effectively for DNA prepared from FFPE breast tumor samples.

Under an approved internal review board protocol for this study, we retrieved FFPE samples of 39 invasive breast carcinomas from the University of Mississippi Medical Center repository. The ages of the deidentified samples ranged from a few months to 5 years. All of the samples included in the study had previously been assessed for HER2 status with IHC and/or FISH in accordance with the ASCO/CAP guideline recommendations. For each sample, we extracted DNA from 4 sections 5 μm thick. Histologic examinations confirmed that each section contained at least 30% tumor cells. DNA was extracted and purified with the QIAamp DNA FFPE Tissue Kit (Qiagen). Purified DNA was quantified with a NanoDrop 1000 spectrophotometer; 0.3–6.0 μg DNA was recovered per section.

ddPCR was performed as previously described *(17, 18)*, with one exception. Given that FFPE DNA is highly degraded, we omitted the restriction enzyme treatment. This treatment is typically performed with relatively pristine DNA to separate linked copy regions for their random segregation into different droplets. In brief, each 20-μL reaction mixture contained 50 ng (3 μL) DNA, 2× ddPCR SuperMix for Probes (Bio-Rad Laboratories), *ERBB2* (*HER2*: Hs02803918 cn; Applied Biosystems), and CEP17 assays. The *ERBB2* assay was purchased as a 20× premix of primers and FAM-MGBNFQ probe and used at 1× concentration. The CEP17 reference assay targets a highly conserved region, which is present at 1 copy per haploid genome and close to the repeat region near the centromere of chromosome 17, which is frequently targeted in FISH analysis. The 1× concentration of this assay comprised 900 nmol/L forward primer (5′-GCTGATGATCATAAAGCCACAGGTA-3′), 900 nmol/L reverse primer (5′-TGGTGCTCAGGCAGTGC-3′), and 250 nmol/L probe (VIC-TGCTGCAATAGGCGG-MGBNFQ), where VIC is a proprietary fluorescent dye (Life Technologies), MGB is a minor groove binder, and NFQ is a nonfluorescent quencher.

The PCR reaction mixtures were partitioned into an emulsion of approximately 20 000 uniformly sized droplets (approximately 1-nL volume per droplet). The droplets were transferred to a 96-well PCR plate, heat sealed, and placed in a conventional thermal cycler (Eppendorf). Thermal cycling conditions were 95 °C for 10 min, 40 cycles of 94 °C for 30 s and 60 °C for 60 s, 98 °C for 10 min, and a 12 °C hold. After the PCR, the PCR plate was loaded on a QX100 droplet reader (Bio-Rad). Analysis of the ddPCR data was performed with QuantaSoft software (version 1.2.10; Bio-Rad). ddPCR results were expressed as the *HER2* copy number (measured concentrations of *HER2* per CEP17 were multiplied by 2 to express copy number on a per cell basis).

The *HER2* status of the 39 cases of invasive breast carcinoma included in this study had previously been determined in accordance with ASCO/CAP Guideline Recommendations for HER2 Testing *(5)*. Of the 39 samples, 36 were tested by IHC, with 23 samples testing negative, 7 testing positive, and 6 being equivocal. The 6 equivocal samples and 12 additional samples were tested by FISH. According to the IHC and FISH data, 10 of the carcinoma samples were identified as positive for HER2, and 29 were identified as negative. The frequencies of the positives and negatives according to the methodology used were 7 and 14, respectively (IHC only), 1 and 2 (FISH only), and 2 and 13 (combined IHC and FISH) (Fig. 1).

The results of the ddPCR analysis for the 39 breast carcinoma samples are summarized in Fig. 1 along with their assignments by IHC and FISH. The samples are arranged left to right in order of increasing ddPCR copy number. Sample 26 exhibited the lowest number of *HER2* copies (1.5), and sample 17 exhibited the highest (75.4). The ddPCR positive-threshold value for *HER2* status was set at 4.4 copies per diploid genome to

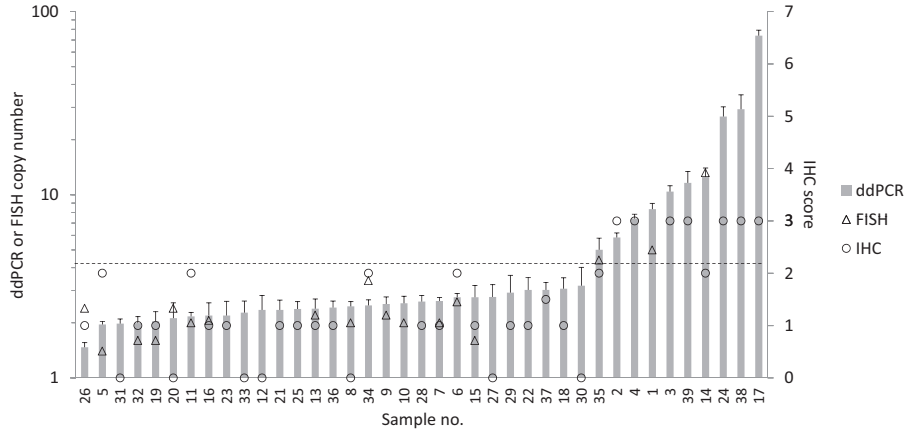

Fig. 1. Summary of ddPCR, IHC, and FISH results for FFPE tissue samples from 39 breast carcinomas.

Each ddPCR copy number value represents the total events (positive and negative droplets) merged from triplicate reactions for each sample. Error bars are based on the DerSimonian–Laird random-effects model. The dashed horizontal line indicates the ddPCR and FISH threshold cutoff of 4.4 copies (based on the 2.2 *HER2*/CEP17 ratio guidance for FISH) for calling a sample HER2 positive. FISH values are expressed as copy number (*HER2*/CEP17 ratio multiplied by 2) to be consistent with ddPCR results. The dashed horizontal line also demarcates IHC-positive (IHC 3+) from IHC-equivocal (2+), and IHC-negative (0, 1+) samples. Four samples had less-defined scores recorded by IHC and FISH: sample 34 (negative by FISH at <3.6 copies), sample 35 (positive by FISH at ≥4.4 copies), sample 36 (negative by IHC at 0–1+), and sample 37 (negative by IHC at 1+ to 2+).

be consistent with the recommended *HER2*/CEP17 threshold ratio of 2.2 per haploid genome established by FISH analysis. On the basis of this threshold criterion, 10 samples (nos. 1–4, 14, 17, 24, 35, 38, and 39) were identified as positive for *HER2* by ddPCR and were distinguishable from the 29 remaining samples that tested negative for *HER2*. The ddPCR-positive *HER2* samples also scored positive by IHC and/or FISH. Samples 2–4, 17, 24, 38, and 39 were IHC positive (i.e., 3+), sample 1 was FISH positive, and samples 14 and 35 were IHC equivocal (i.e., 2+) and FISH positive. The results for samples 1, 14, and 35 (ddPCR copy number measurements of 8.4, 12.5, and 5.0, respectively) were consistent with their corresponding FISH copy number values (*HER2*/CEP17 ratio multiplied by 2) of 5.0, 13.2, and >4.4, respectively. The ddPCR results agreed with the FISH results for the 6 cases that were equivocal by IHC, thereby confirming 2 of these samples as positive for *HER2* and the other 4 as negative. The 29 *HER2*-negative samples displayed ddPCR copy numbers from 1.6 to 3.1, within the range of the values assigned to the 15 negatively testing samples analyzed by FISH. Thus, ddPCR correctly identified the 39 breast carcinoma cases: 10 *HER2* positive (26%) and 29 *HER2* negative (74%), for a 100% concordance with the IHC and FISH results.

In summary, somatic copy number alteration is the hallmark of many cancers. FISH is currently the "gold standard" for diagnosing amplifications and deletions in clinical samples, because this technique affords single-cell resolution. FISH and related histopathology techniques (such as IHC) are laborious and subject to potential losses in performance, owing to other analytical factors *(19)*. Furthermore, evaluating results with these microscopy-based techniques can be subjective, introducing the possibility that different pathologists could characterize the same cancer differently. Our work demonstrates that ddPCR can be used as a molecular-analysis tool to precisely measure *HER2* copy number alterations in FFPE samples of heterogeneous breast tumors at both the RNA and DNA levels.

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