Noninvasive Detection of HER2 Amplification with Plasma DNA Digital PCR

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Abstract

Purpose: Digital PCR is a highly accurate method of determining DNA concentration. We adapted digital PCR to determine the presence of oncogenic amplification through noninvasive analysis of circulating free plasma DNA and exemplify this approach by developing a plasma DNA digital PCR assay for HER2 copy number.

Experimental Design: The reference gene for copy number assessment was assessed experimentally and bioinformatically. Chromosome 17 pericentromeric probes were shown to be suboptimal, and EFTUD2 at chromosome position 17q21.31 was selected for analysis. Digital PCR assay parameters were determined on plasma samples from a development cohort of 65 patients and assessed in an independent validation cohort of plasma samples from 58 patients with metastatic breast cancer. The sequential probability ratio test was used to assign the plasma DNA digital PCR test as being HER2-positive or -negative in the validation cohort.

Results: In the development cohort, the HER2:EFTUD2 plasma DNA copy number ratio had a receiver operator area under the curve (AUC) = 0.92 [95% confidence interval (CI), 0.86–0.99, P = 0.0003]. In the independent validation cohort, 64% (7 of 11) of patients with HER2-amplified cancers were classified as plasma digital PCR HER2-positive and 94% (44 of 47) of patients with HER2-nonamplified cancers were classified as digital PCR HER2-negative, with a positive and negative predictive value of 70% and 92%, respectively.

Conclusion: Analysis of plasma DNA with digital PCR has the potential to screen for the acquisition of HER2 amplification in metastatic breast cancer. This approach could potentially be adapted to the analysis of any locus amplified in cancer. Clin Cancer Res; 19(12); 3276–84. ©2013 AACR.

Introduction

Genomic amplifications present important therapeutic targets as shown by the efficacy of the HER2 targeting antibody trastuzumab in patients with HER2 (ERBB2)-amplified breast and gastric cancers (1, 2). In routine clinical practice, the presence of an amplification is determined by analysis of a tumor biopsy at initial diagnosis. However, amplifications can be "acquired" and lost, through tumor progression and prior treatment (3, 4), and this presents a substantial challenge to the concept of personalized cancer therapy. For example, HER2 amplification is "acquired" in about 2% to 5% of metastatic breast cancers that originally had HER2-nonamplified primary cancers (4). MET amplification may be "acquired" as a mechanism of resistance to EGF receptor (EGFR) inhibitor therapy in non–small cell lung cancer (5), and amplification of c-MYC may be a common mechanism of resistance to many targeted therapies (6). The underlying biology behind "acquisition" of amplifications, at least in part, reflect intratumor heterogeneity and clonal selection (7).

To optimally deliver targeted therapy, repeated sampling of a tumor is therefore required to determine whether the genetic profile of a cancer has altered following prior therapy. In current practice, this would require repeated biopsies of recurrent and metastatic cancers, yet this approach has limitations. Biopsy has associated risks and may be technically challenging depending on the site(s) of relapsed cancer. Biopsy usually samples only a single area of tumor, and in heterogeneous tumors, may underestimate the array of genetic aberrations present (8). Ideally, to overcome these limitations and to allow repeated sampling, the presence of amplification could be diagnosed noninvasively.

DNA arising from tumor cells is found in the plasma of patients with cancer and this represents a potential source of...
noninvasively analyzing tumor DNA (9). Indeed, high sensitivity assays of coding mutations on plasma DNA, also referred to as cell-free DNA or circulating free DNA, have reported high concordance with cancer mutational status (10, 11). Assays of plasma DNA are noninvasive, can be repeated at multiple occasions throughout the disease course, and potentially may assess the full heterogeneity of mutations present. Analysis of plasma DNA requires an assay of high sensitivity as DNA is frequently present at only low concentration in plasma and tumor cell–derived DNA may be only a small fraction of the total plasma DNA (9, 12), with the remainder being derived from somatic cells.

Digital PCR has the potential to highly accurately quantify the concentration of nucleic acids in a sample, to a much greater degree than traditional quantitative PCR, through counting individual DNA molecules (13). We investigated whether digital PCR could be adapted to detect small increases in plasma DNA gene copy number that accompany a cancer-specific amplification, in a similar fashion to the diagnosis of fetal aneuploidy on the basis of maternal plasma DNA analysis (14). To examine the potential of digital PCR for amplification detection we developed an assay for HER2, ultimately showing that digital PCR has high concordance with tumor-derived HER2 status in an independent validation set.

Materials and Methods

Patient cohort

Blood samples were obtained from a consecutive prospective series of patients with metastatic breast cancer treated at the Royal Marsden Hospital between 2010 and 2012. All patients had recently progressed following prior therapy. Patients were allowed to be taking maintenance therapies such as hormone therapy or trastuzumab at the time of plasma sampling. Estrogen receptor (ER), progesterone receptor (PR), and HER2 were assessed in a single laboratory at the Royal Marsden Hospital Histopathology department. A tumor was considered to be HER2-positive if 3+ positive by Hercept test or 2+ positive with a FISH/SISH HER2:CEP17 ratio ≥ 2.2 (15). For patients who had biopsy of recurrent cancer, pathology of the recurrent cancer biopsy was compared with digital PCR, and for the other patients, the pathology of the original primary cancer was used. Patients who presented primary breast cancer simultaneously with metastatic disease were recorded as having biopsy of recurrent cancer. Research was approved by the Royal Marsden Hospital Research Ethics Committee.

Identification of reference region on chromosome 17

We used microarray comparative genomic hybridization data from 311 invasive breast cancers, 65 HER2-amplified, and 246 HER2-nonamplified to identify an optimal chromosome 17 copy number reference region (16). The copy number ratio between the mean of all probes covering EBBB2 (HER2) and every BAC probe on chromosome 17 was assessed for each cancer. For each BAC probe, the sensitivity comparing HER2-amplified and -nonamplified cancers was calculated, as was the statistical significance of the difference between HER2-amplified and -nonamplified cancers with the Student t test. The sensitivity was assessed as the proportion of HER2-amplified cancers that had a copy number ratio higher than the maximum ratio of the HER2-nonamplified cancers. All genomic positions were according to genome version hg19. TCGA data was from SNP pipeline 3.0 data from the BROAD institute (www.broadinstitute.org/).

Plasma DNA collection and quantification

Plasma was collected in CPT tubes (BD Biosciences) and centrifuged within 2 hours of venesection. DNA was extracted with QIAamp MinElute virus spin kit (Qiagen) essentially according to manufacturer’s instruction and quantified as described in Supplementary Methods.

Digital PCR

Digital PCR for HER2:UBBP4 was conducted in 384-well format as discussed in Supplementary Methods. Digital PCR for HER2:EFTUD2 was conducted with the Bio-Rad QX100 system using custom primers against HER2 and EFTUD2 reference. DNA was diluted to aim for about 400 copies per well and partitioned into about 14,000 droplets as per manufacturer instructions. PCR reactions were run on G-Storm GS4 thermal cycler incubating the plates at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds, followed by 10-minute incubation at 98°C. Plates were read on a Bio-Rad QX100 droplet reader using QuantaSoft v1.2.10.0 software from Bio-Rad to assess the number of droplets positive for HER2, EFTUD2, both or neither.

Digital PCR analysis development cohort

Assessment of pericentromeric UBBP4 and TUFML probes is described in Supplementary Methods. HER2: EFTUD2 copy number ratio droplet digital PCR was analyzed in the development cohort by calculating the copies per droplet from the Poisson distribution. We aimed for at least 400 droplets positive for EFTUD2 to accurately assess the ratio. The development cohort was analyzed with a receiver operator curve.
Digital PCR analysis of independent validation cohort with sequential probability ratio test

The validation cohort was evaluated using the sequential probability ratio test (SPRT) with a likelihood ratio of 8, as previously reported with modifications (14, 17). The thresholds for the SPRT were assessed from the development cohort before prospective analysis of the validation cohort. For the SPRT, only informative droplets were analyzed, those droplets positive for either HER2 alone or control probe alone. Analysis with SPRT is discussed in detail in Supplementary Methods. Samples were assessed blinded to HER2 amplification status.

Results

Pericentromeric probes are suboptimal for HER2 digital PCR assessment

To assess the potential of plasma DNA digital PCR, we optimized an assay for HER2 copy number. HER2 copy number in digital PCR is assessed relative to a reference gene, and we initially assessed 2 potential pericentromeric genes adjacent to the chromosome 17 centromere (CEP17), centromeric DNA itself consisting predominantly of highly repetitive α-satellite DNA not suited to the design of a specific PCR amplicon. We designed and optimized a set of custom primer probes using TaqMan MGB chemistry (Applied Biosystems), with an HER2 probe labeled with FAM and reference probes labeled with VIC. We selected reference probes through avoidance of known single-nucleotide polymorphisms and regions of normal copy number variation (as discussed in Supplementary Methods). The 17q pericentromeric gene TUFML was rejected as a reference probe due to co-amplification of 17q pericentromeric DNA in some HER2-amplified cancers (Fig. 1A), and we therefore assessed the 17p centromeric gene UBBP4.

For the assessment of HER2:UBBP4 ratio, we used 384-well microplate digital PCR (Supplementary Figs. S1 and S2). We first determined the optimal threshold to define an elevated plasma DNA Digital PCR HER2:UBBP4 ratio in a development set of plasma samples taken from patients with metastatic breast cancer. Although a HER2:CEP17 ratio of ≥2.0 is used when directly analyzing tumor DNA, for example, by FISH, a lower threshold is required in the analysis of plasma DNA as tumor-derived DNA is only a small fraction of total plasma DNA, with the majority being derived from stromal/normal gDNA (12). In the development set of 44 patients (described in Supplementary Table S1), the digital PCR HER2:UBBP4 ratio in patients with HER2-amplified cancers (median, 2.231; range,
1.138–7.89) was significantly elevated compared with HER2-nonamplified cancers (median, 1.046; range 0.70–1.245; \( P < 0.001 \); Mann–Whitney U test; Fig. 1B), with an ROC curve AUC 0.967 [95% confidence interval (CI), 0.585–0.997]. A threshold to define an HER2-positive cancer was selected as 1.25 in digital PCR to accommodate a small decrease in specificity.

We assessed the assay in an independent validation cohort of 46 patients (Supplementary Table S1). In the validation set, we used the SPRT to assign a sample as being from a patient with HER2-amplified (HER2-positive) or nonamplified cancer (HER2-negative). The SPRT uses Bayesian likelihood methods to assess whether after each round of digital PCR the results should be assigned as HER2-positive, HER2-negative, or unassigned requiring further rounds of digital PCR to determine the HER2 status (Supplementary Fig. S1). We used only informative wells in the SPRT, as the informative wells ratio modestly amplifies small ratios (14). The SPRT parameters were set with a likelihood ratio of 8 to differentiate between a ratio of 1.3 as HER2-positive and 1.2 as HER2-negative (corresponding to the 1.25 threshold, Supplementary Methods).

In the independent validation cohort, of the patients with HER2-amplified cancers 80% (8 of 10) were classified as digital PCR HER2-positive, and with HER2-nonamplified cancers, 75% (27 of 36) were classified as digital PCR HER2-negative (Fig. 1C, \( P = 0.003 \); Fisher’s exact test) providing a highly significant proof of principle. However, the specificity of 75% and positive predictive value of 47%, questioned the potential clinical usefulness of such an assay based on the UBBP4 pericentromeric probe. Loss of chromosome arm 17p occurs in about 10% to 15% of HER2-nonamplified cancers as a result of genomic chromosomal instability (16, 18). The resulting loss of one copy of UBBP4 in the tumor may cause a false-positive due to elevation of the \( \text{HER2:UBBP4} \) ratio not because of HER2 gain but due to UBBP4 loss.

**Identification of a superior chromosome 17 reference probe**

We bioinformatically examined for a potentially superior copy number reference region on chromosome 17. An optimal reference region would be one that in HER2-amplified cancers is never coamplified with HER2 and in nonamplified cancers robustly has the same stable copy number as HER2. We examined publically available microarray comparative genomic hybridization (array CGH) profiles of 311 invasive breast cancers (16). We assessed the ERBB2-

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**Figure 2. Identification of an optimal chromosome 17 reference probe for digital PCR assay.**

A, analysis of microarray CGH data from 311 primary breast cancers (16). For each cancer, the ERBB2 (HER2) to reference copy number ratio was calculated for every genomic position along chromosome 17. The ERBB2 to reference copy number ratio of HER2-amplified cancers was compared with HER2-negative cancers using Student \( t \) test. Displayed is the \( \log_{10} \) \( P \) value for each genomic position, with arrow indicating the locus of EFTUD2 with the most significant difference between amplified and nonamplified cancers. B, the corresponding sensitivity for each reference genomic position was assessed for each genomic position. C, comparison of ERBB2:EFTUD2 and ERBB2:UBBP4 copy number ratios in HER2-amplified and -nonamplified cancers from the same micro-array CGH series (16). D, correlation of ERBB2 and EFTUD2, along with ERBB2 and UBBP4, copy number in the 246 HER2-nonamplified cancers. The copy numbers of ERBB2 and EFTUD2 are highly correlated as low level gain or loss of ERBB2 extends to EFTUD2. EFTUD2 therefore is predicted to generate stable copy number ratios in the analysis of nonamplified cancers.
(HER2) to reference copy number ratio for every genomic position across chromosome 17 and examined for the genomic region that gave the most significantly different copy number ratios between HER2-amplified and -nonamplified cancers and that had the highest sensitivity (Fig. 2A and B).

A region on chromosome 17q21.31 from about 42.2 to 43.9 Mb was identified as the optimal region by this assessment, about 5 Mb telomeric of the ERBB2 (HER2) locus. This region was coamplified with the ERBB2 locus in none of the 65 HER2-amplified cancers in the series. The EFTUD2 gene was selected in this region (42.93–42.98 Mb) as not being subject to normal copy number variation (Supplementary Methods). In HER2-nonamplified cancers, the EFTUD2 locus had a highly stable copy number ratio with the ERBB2 locus (Fig. 2C and D), to a substantially greater extent than UBBP4 (Fig. 2C and D), and therefore EFTUD2 would be anticipated to have more consistent copy number ratio in nonamplified cancers.

We analyzed SNP copy number data from the TCGA data set and confirmed that EFTUD was coamplified with ERBB2 in none of the 110 cancers with focal ERBB2 amplification (19). Therefore, considering both data sets, EFTUD2 was coamplified with ERBB2 in none of 175 HER2-amplified cancers.

Digital PCR with EFTUD2 control probe has high diagnostic accuracy

We determined the parameters of a HER2:EFTUD2 digital PCR assay. For this, we switched to a specific digital PCR platform, due to the development of relatively inexpensive dedicated platforms, that partition a PCR reaction into about 14,000 droplets (20). Following PCR, each droplet is individually assessed by a fluorescent reader to effectively assay about 14,000 individual PCR reactions (Fig. 3 and Supplementary Fig. S3). The assay parameters for a plasma HER2:EFTUD2 assay were determined in a development set of 65 patients (described in Table 1). Analysis of the development set by ROC had an AUC 0.92 (95% CI, 0.86–0.99, P = 0.0003; Fig. 4A). Assessing the 58 patients with HER2-nonamplified cancer in the development set, we selected a cutoff of 1.25 as this was estimated to give a specificity of about 95%.

The HER2:EFTUD2 plasma DNA test was assessed in an independent prospectively collected validation cohort of 58 patients (Supplementary Fig. S4). The SPRT was again used...
to analyze the validation set, with a likelihood ratio of 8 to differentiate between HER2:EFTUD2 ratio of 1.3 as HER2-positive and 1.2 as HER2-negative (corresponding to the 1.25 threshold). Of the patients with HER2-amplified cancers, 64% (7 of 11) were classified as plasma DNA digital PCR HER2-positive, and of the patients with HER2-non-amplified cancer, 94% (44 of 47) were classified as plasma DNA digital PCR HER2-negative ($P = 0.0001$, Fisher exact test; Fig. 4B and C). The positive predictive value was 70% and negative predictive value 92%.

**Discussion**

We have shown that analysis of plasma DNA with digital PCR has high accuracy in the determination of HER2 status. An assay based on the plasma DNA HER2:EFTUD2 ratio had a high concordance rate of 90% with tumor-derived HER2 status in an independent validation set. This degree of concordance is similar to that reported between local and central laboratories for routine clinical HER2 testing on biopsy material (21) and would potentially be sufficient to use this test to screen for cancers that have acquired HER2 amplification in the metastatic setting. The potential clinical use of such a test would be in assessing patients who have not had biopsy of recurrent breast cancer as part of their routine care. Although biopsy of recurrent disease is a standard of care, to confirm diagnosis and reassess hormone receptor and HER2 status, recurrent disease is in routine clinical practice frequently not biopsied when such a biopsy is technically challenging and there is no diagnostic doubt that relapse has occurred. A noninvasive test to screen for acquisition of HER2 amplification in such circumstances would have potentially high clinical use.

Breast cancers are frequently chromosomally unstable and this presents a challenge to the development of a plasma DNA digital PCR assay for HER2. To achieve high accuracy, HER2-positive cancers with a low fraction of tumor-derived DNA in plasma (plasma DNA highly diluted with normal germline DNA) must be discriminated from aneuploid HER2 negative cancers with a high fraction of tumor-derived DNA in the plasma. The factors that are required to discriminate between these 2 scenarios are a

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<th>Table 1. Clinicopathologic characteristics of patients included in study</th>
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<td>Prior HER2-directed therapy</td>
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Abbreviations: IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; No prior metastatic chemo, number of prior courses of chemotherapy in the metastatic setting.
reference probe that has a highly stable copy number with HER2 in nonamplified cancers and yet is robustly not amplified with HER2. In assessing patients with nonamplified cancers, a reference probe with a highly stable copy number to HER2 will robustly have a copy number ratio of 1 regardless of the fraction of tumor-derived DNA in the plasma. We describe an approach to bioinformatically identify a reference probe with these characteristics, exploiting the well-described genomic landscape of breast cancer, and show improved specificity of the identified EFTUD2 region over pericentromeric probes (Supplementary Figs. S5 and S6). RNase P (RPPH1) on chromosome 14q11.2 is frequently used as a reference probe in assays of normal copy number variation, as it is not subject to normal copy number variation. However, using a control probe from a different chromosome will not deliver optimal characteristics for plasma DNA analysis in aneuploid cancers where highly accurate discrimination in copy number is required (Supplementary Fig. S5).

The results we report show substantially higher concordance with tumor-derived HER2 status compared with prior reports of noninvasive approaches. Quantitative real-time PCR assessment of HER2 copy number in plasma DNA has only low concordance (22). Similarly, studies assessing HER2 status by immunofluorescence or FISH on circulating tumor cells (CIC) have reported a substantial positive CTC rate in originally HER2-negative cancers (23, 24), although more recent studies with strict cutoffs report a higher level of concordance (25). No studies have yet compared CTC HER2 assessment with digital PCR to establish which is the more robust way of identifying cancers that may derive benefit from HER2 targeting in the metastatic setting. Other
techniques such as massive parallel sequencing of circulating free DNA may present another potential method for detection of amplifications (26), although the application of such technology to plasma DNA is at an early stage. BEAMing, which has been used for mutation detection in plasma (10, 27), could also be adapted for copy number detection. Although tumor-derived DNA can be detected in the plasma of patients with early breast cancer, or without apparently overt metastatic disease (28), in the majority of patients insufficient plasma DNA is present to allow for the formal digital PCR analysis we describe (data not shown).

For the cases of discordance between tumor-derived HER2 status and plasma DNA digital PCR, we reassessed HER2 status by in situ hybridization on the corresponding tumor samples (Supplementary Table S2). Of the 4 patients who were originally defined as HER2-amplified in the cancer, but were HER2 negative by plasma DNA digital PCR (Fig. 4C), one tumor was not HER2-amplified on retesting (Supplementary Table S2). This suggested that this case may have been correctly called negative by digital PCR assay and therefore that the positive predictive value of the test may be higher than reported in the validation cohort. Interesting a further false-negative patient had a positive but low level of HER2 amplification (Supplementary Table S2).

It will be interesting in future research to ascertain the reasons for discordance between tumor HER2 status and plasma DNA digital PCR status. The 3 patients with apparent false positive plasma digital PCR tests in the validation set may represent genuine false-positive tests due to low level loss of EFTUD2 combined with a high fraction of tumor cell–derived DNA in the plasma. Alternatively, these patients may possibly also have acquired HER2 amplification. Interestingly in the development set, one HER2-non-amplified cancer was classified as unequivocally digital PCR HER2–positive, with a HER2:EFTUD2 ratio of 16.9 (Supplementary Fig. S6). This was confirmed on a separate plasma sample from the patient with the same result (data not shown). For this patient, tumor HER2 status had been determined on her original primary cancer, as her metachronous metastatic cancer had not been biopsied as part of her routine care. Considering the 3 patients with apparently false-negative digital PCR tests, again the discordance might reflect a genuine change in HER2 status. All patients with apparently false-negative plasma DNA tests had received prior HER2-directed therapy in the metastatic setting, that might in tumors with intratumoral heterogeneity select for outgrowth of a nonamplified clone (29). However, a potential other explanation would be a very low percentage of tumor-derived DNA content in plasma DNA, combined with a relatively low level of HER2 amplification, causing a genuine false-negative test.

In this study, we show that digital PCR of plasma DNA has high accuracy in the determination of HER2 status. This approach could be adapted to the assessment of any amplified locus in cancer and in particular may be a useful strategy screening for potentially rare acquisition events in response to therapy, such as acquisition of MET amplification following EGFR targeting therapy in lung cancer (5). Combined with sensitive coding mutation assays, our data suggest that it may be possible to replace routine repeat biopsies of metastatic cancer for the optimal delivery of targeted therapies.

Disclosure of Potential Conflicts of Interest
I.E. Smith has received honoraria from speakers’ bureau and is a consultant/advisory board member of Roche. N.C. Turner is a consultant/advisory board member of Roche. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Gevensleben, I. Garcia-Murillas, M.K. Graeser, P. Osin, I.E. Smith, A. Ashworth, N.C. Turner
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Gevensleben, I. Garcia-Murillas, P. Osin, N.C. Turner
Writing, review, and/or revision of the manuscript: I. Garcia-Murillas, I.E. Smith, A. Ashworth, N.C. Turner
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Study supervision: N.C. Turner
Identification of suitable patients and recruitment to study: M. Parton

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