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CLINICAL TRIAL

Use of a multiplexed immunoassay (PRO Onc assay) to detect HER2 abnormalities in circulating tumor cells of women with HER2-negative metastatic breast cancer: lack of response to HER2-targeted therapy

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Abstract
Purpose Determination of HER2 status by testing circulating tumor cells (CTCs), compared to sampling tumor biopsies, may improve patient management by allowing ongoing assessment of HER2 status during the disease course. The PRO Onc assay (Prometheus Laboratories; San Diego, CA) is a multiplexed immunoassay that measures the expression and activation of HER2 in CTCs. In this study, we screened patients with metastatic HER2-negative breast cancer with the PRO Onc assay; patients with HER2 overexpression or activation received a trial of HER2-targeted therapy.
Methods In Part 1 of the trial, patients with HER2-negative breast cancer were screened with the PRO Onc assay to confirm the presence of a cohort that tested HER2-positive. After this finding was confirmed, patients in Part 2 of the study with HER2 abnormalities received a trial of treatment with trastuzumab/pertuzumab.
Results In Part 1, 31 of 57 specimens contained CTCs; of these, 12 (38 %) showed HER2 abnormalities by PRO Onc assay. In Part 2, 129 of 226 patients (57 %) had CTCs; 24 of these patients (19 %) had HER2 abnormalities detected. Fourteen patients were treated with HER2-targeted therapy. Twelve of 14 patients progressed within 6 weeks, one patient had a brief (12 weeks) partial response, and one patient was stable for 12 weeks.
Conclusions HER2 overexpression or activation was detected by the PRO Onc assay in 22 % of HER2-negative patients with CTCs. However, HER2-targeted therapy was not effective in such patients. FISH and IHC staining remain the standards for HER2 determination.

Keywords HER2-negative · HER2 abnormalities · PRO Onc assay

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Introduction

The development of effective HER2-targeted therapy has markedly improved survival in the 20–25 % of breast cancer patients with HER2-positive tumors [1–4]. At present, FISH analysis is considered the "gold standard" for identification of HER2-positive tumors; 3+IHC staining correlates highly with FISH positivity and is used as a surrogate measurement [5]. In spite of the reliability of FISH testing, there are several scenarios in which a false-negative result could be obtained. First, some HER2-positive breast cancers may have levels of HER2 amplification which are below the level detectable by the FISH assay. Second, HER2 phosphorylation may occur, with activation of downstream signaling, in the absence of HER2 overexpression. Such patients may respond to intracellular HER2-targeted agents such as lapatinib, or to treatment with pertuzumab [6, 7]. Finally, HER2 expression in metastases is sometimes discordant with HER2 expression measured in the primary tumor [8–10]. Since HER2 status is usually documented once (at the time of diagnosis),

patients who subsequently develop HER2-positive metastases may not be recognized.

Recently, methods have been developed that enable detection and measurement of molecular abnormalities in cancer cells by assaying tumor DNA obtained from circulating tumor cells (CTCs) or plasma. Since these assays can be performed on blood specimens, they offer several potential advantages over the current assays performed on tumor biopsy specimens. The PRO Onc assay (Prometheus, Inc.) uses a multiplexed immunoassay format, and can provide expression and activation profiling of HER2 and other signal transduction pathway molecules [11]. This assay can detect both overexpression and activation (i.e., phosphorylation) of HER2 using small amounts of target protein obtained from fine-needle aspiration biopsies or CTCs. In early clinical testing, detection of HER2 in CTCs correlated with positive results obtained by standard methods [11, 12]. However, HER2 overexpression and/or phosphorylation were also detected in a minority of patients whose cancers did not show overexpression by IHC [12].

The current study was designed to evaluate the clinical utility of the PRO Onc assay, with particular focus on the identification of a population of HER2-positive patients previously classified as HER2-negative by standard assay methods (FISH, IHC). In Part 1, the PRO Onc assay was performed on blood specimens from patients with HER2-negative metastatic breast cancer (MBC) to determine the frequency of HER2-positive results in this population. Part 2 of the study evaluated the efficacy of HER2-targeted therapy in this patient population.

Patients and methods

Enrollment to this trial began in February 2011. Part 1 of the trial was completed at a single site (Tennessee Oncology, Nashville, TN); 6 sites participated in Part 2. The institutional review board of each site approved the study prior to patient enrollment and was conducted in accordance with the Declaration of Helsinki. All patients provided written consent to participate.

Eligibility

Women with HER2-negative MBC who had received at least one previous chemotherapy regimen were eligible. HER2-negativity was defined by either negative FISH testing, or by negative IHC staining (0 or 1+). In patients who were HER2-negative on the basis of IHC alone, subsequent FISH testing was required if the PRO Onc assay demonstrated HER2-positivity. Additional entry requirements included ECOG performance status 0, 1, or 2; life expectancy >12 weeks. Patients were excluded if they were considered unlikely candidates for continued MBC treatment following their next episode of cancer progression. Additional exclusion criteria: previous treatment with HER2-targeted agents; meningeal metastases; congestive heart failure or recent ischemic cardiac events; and concurrent severe medical illnesses.

In Part 2 of the study, patients eligible for HER2-targeted treatment were also required to have measurable disease (RECIST version 1.1 [13]), as well as adequate bone marrow, liver, and kidney function. Patients with active brain metastases were excluded.

All patients were required to understand the investigational nature of this study, and to give written informed consent prior to study entry.

Study design

In Part 1 of the study, a single blood sample was obtained and sent to Prometheus where CTCs were isolated using the Cellsearch CTC Test (Veridex). Enriched CTCs were then activated, lysed, and stored at −80 °C until the PRO Onc assay was performed. No protocol-directed treatment was given in this part of the study. Patients continued their standard treatment for HER2-negative breast cancer.

The Part 2 study design is diagrammed in Fig. 1. Blood samples were obtained for the PRO Onc assay, as in Part 1 of the study. Patients who had HER2 overexpression or activation detected by the PRO Onc assay were eligible to receive treatment with HER2-targeted therapy at the time of their next breast cancer progression, if they still met all entry criteria at that time. Patients in Part 1 who were HER2-positive by PRO Onc assay were also eligible to participate in Part 2, assuming they still met eligibility criteria.

PRO Onc assay

The PRO Onc assay was performed by Prometheus Inc. using methods previously described [11, 12]. Briefly, the assay is a multiplexed, collaborative proximity immunoassay using a COPIA platform. The assay platform is built on a triple-antibody microarray system: one capture antibody binds the target protein to the microarray surface, while two detector enzyme-conjugated antibodies co-localize to form a unique immunocomplex, which activates the channeling events between two detector enzymes in proximity (Fig. 2). The assay therefore allows the profiling of the receptor tyrosine kinase with extreme sensitivity.

Patients were defined as having HER2 overexpression or activation when the PRO Onc assay showed values >3 CU

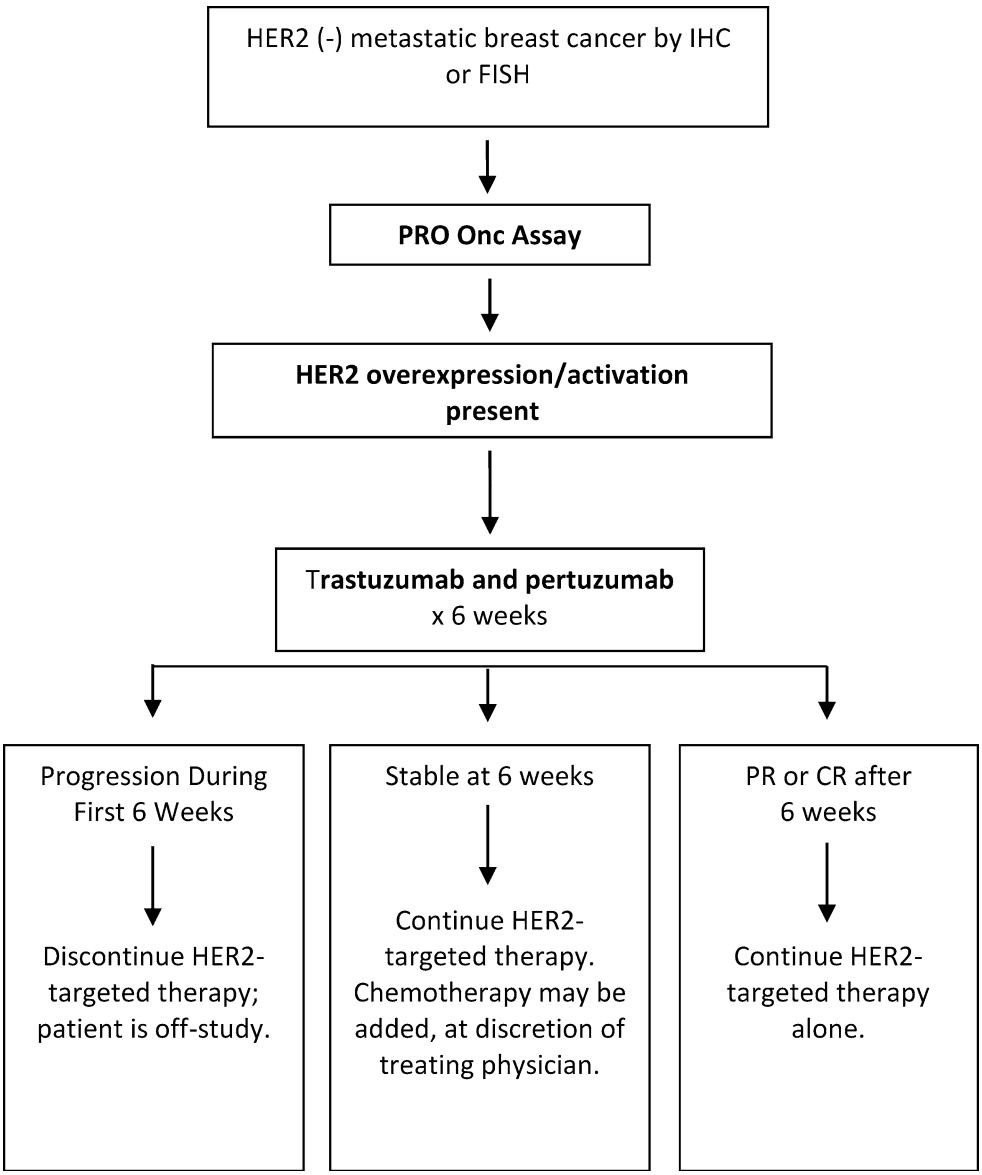

Fig. 1 Study design for participants in Part 2

or >0.41 CU, respectively. These cutoff values were arbitrarily selected as exceeding the reference mean value (determined from testing blood specimens from normal volunteers) by four standard deviations.

Pretreatment evaluation

Patients in Part 2 of the study who were found to be candidates for HER2-targeted therapy were evaluated with medical history, physical examination, complete blood counts, metabolic profile, blood coagulation studies, and left ventricular ejection fraction determination (MUGA scan or echocardiogram). Tumor status was quantified using computed tomography of the chest and abdomen, performed within 4 weeks of the initiation of study treatment.

Treatment

In Part 2, patients who were HER2-positive by the PRO Onc assay and met all entry criteria received treatment with trastuzumab (8 mg/kg IV loading dose, followed by 6 mg/kg every 3 weeks) and pertuzumab (840 mg IV loading dose, followed by 420 mg every 3 weeks). Both agents were administered according to standard guidelines. Dose modifications for treatment-related toxicity also followed standard guidelines.

Determination of response

After completion of two cycles (6 weeks) of treatment, patients were evaluated for response. Patients with stable disease or objective response (RECIST version 1.1)

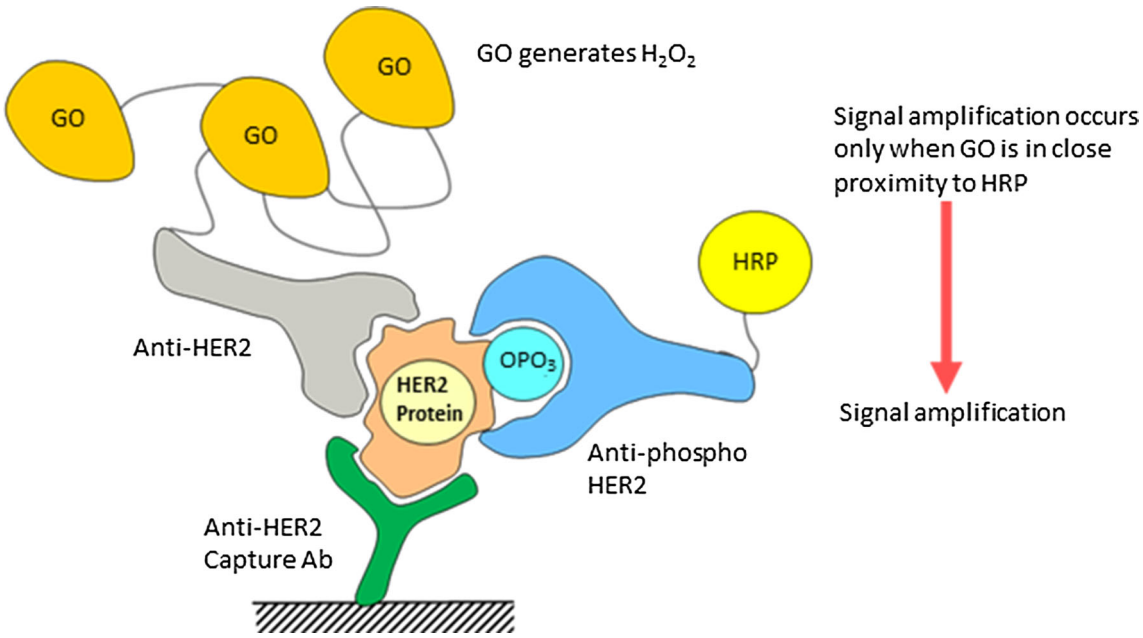

Fig. 2 Diagram of the PRO Onc assay. The platform uses a triple-antibody microarray system: one capture antibody binds the target HER2 protein to the microarray surface, and 2 detection antibodies (Anti-HER2, anti-phospho-HER2) form a unique immunocomplex, activating 2 detector enzymes (glucose oxidase [GO] and horseradish peroxidase [HRP])

continued treatment with trastuzumab and pertuzumab, with subsequent reevaluations at 6-week intervals. In patients with stable disease, chemotherapy could be added to trastuzumab/pertuzumab at the discretion of the treating physician. Patients were removed from treatment if disease progression or intolerable toxicity occurred.

Statistical considerations

The purpose of Part 1 of this trial was to establish the incidence of HER2 overexpression or activation, as determined by the PRO Onc assay, in women with HER2-negative MBC (as determined by standard testing). If the true incidence is >10 %, the PRO Onc assay may have considerable clinical value in this patient population. Assuming an incidence of 20 %, testing a total of 52 samples containing CTCs would provide an estimated incidence with a two-sided confidence interval of ±10 %. We anticipated that CTCs (necessary to perform the assay) would be present in approximately 70 % of patients. Therefore, screening of 74 women was targeted for this portion of the study.

The goal of Part 2 was to determine the response rate to HER2-targeted therapy (trastuzumab+pertuzumab) in patients found to be HER2-positive by the PRO Onc assay. The estimated response rate to trastuzumab in HER2-positive patients receiving second- to fourth-line therapy for MBC is approximately 15 % [14]. Therefore, documentation of a similar response rate in patients identified by the PRO Onc assay would suggest the validity of the assay in detecting a sensitive population. Given these assumptions, a group of 48 treated patients is required to provide a two-sided 90 % confidence interval with a width equal to 0.20. Again, assuming a 70 % rate of CTC identification, screening of 460 patients was necessary in order to identify the target population of 48 patients. Since the utility of the PRO Onc assay was undefined, treatment responses in the first 14 patients were reviewed. If no evidence of treatment activity was identified in these patients, the study could be stopped for futility at the discretion of the principal investigator.

Results

Between February 2011 and June 2014, 283 patients were enrolled in this clinical trial. Fifty-seven patients participated in Part 1. When the incidence of HER2-positivity detected by the PRO Onc assay was found to be greater than the specified target level of 10 %, Part 2 of the study was initiated, and 226 patients were enrolled.

Patient characteristics

Table 1 shows the pertinent clinical characteristics of the patients screened in Part 1 of this study, as well as the composite characteristics of 241 patients who participated. (Complete data were not obtained in 42 of the patients who

Table 1 Patient characteristics ($N = 241$)

Characteristic	Number of patients (%)	
	Part 1 ($N = 57$)	All Patients ($N = 241$)
Median age (range)	58 (27–85)	60 (27–90)
Race		
Caucasian	45 (79 %)	212 (88 %)
African American	12 (21 %)	25 (10 %)
Asian	0	2 (1 %)
American Indian/Alaska Native	0	1 (0.4 %)
Other	0	1 (0.4 %)
Prior systemic treatment regimens for MBC		
1	43 (75 %)	75 (31 %)
2	9 (16 %)	50 (21 %)
≥3	5 (9 %)	115 (48 %)
Unknown	0	1 (0.4 %)
Median (range)	1 (1-6)	2 (1-15)
Hormone receptor status		
ER and/or PR positive	44 (77 %)	176 (73 %)
ER and PR negative	13 (23 %)	63 (26 %)
Unknown	0	2 (1 %)
Previous HER2-negative determination		
FISH	32 (56 %)	131 (54 %)
IHC	24 (42 %)	108 (45 %)
Unknown	1 (2 %)	2 (1 %)
Median interval, initial HER2 testing to PRO Onc Assay, months (range)	36 months (2–144)	36 months (<1–245)*

* Calculation is based on $N = 240$. Date of initial HER2 testing was unknown for one patient

were HER2-negative by PRO Onc assay, and these patients are not included in Table 1). All patients were HER2-negative by standard testing (FISH-negative, 131 patients; IHC-negative, 108 patients). In the large majority of patients, initial HER2 testing was performed using biopsy material from the breast primary site, obtained a median of 48 months prior to the blood sample used for the PRO Onc assay. The median age was 60 years; 63 patients (26 %) were hormone receptor-negative. Patients had received a median of 2 systemic treatment regimens for MBC (range 1–15), and all hormone receptor-positive patients had received at least one hormonal therapy prior to entering this trial. The median interval between the initial HER2 determination and the PRO Onc assay determination was 36 months (range <1–245 months).

Part 1 study results

In Part 1 of the study, the PRO Onc assay was performed on specimens from 57 patients. In 31 specimens (54 %), CTCs were identified. Of the 31 specimens successfully assayed, six tumors (19 %) were found to have HER2 overexpression, and six tumors (19 %) had HER2 activation without overexpression. Therefore, a total of 12 patients (39 % of patients successfully assayed; 21 % of total patients entered) were found to have either HER2 overexpression or activation. Although the target number of 52 specimens with CTCs had not been assayed, the 39 % incidence of HER2 positivity detected by the assay was considered sufficient to proceed to Part 2 of the study.

Summary of PRO Onc assay results, entire study population

Table 2 summarizes the PRO Onc assay results in the 283 patients enrolled in the study. Circulating tumor cells were detected in 165 patients (57 %). In Part 2 of the study, the CTC-positive patients had a median of 11 CTCs isolated (range 1–10,000). A total of 36 patients (13 %) were found to have HER2 abnormalities (overexpression, 19 patients; activation without overexpression, 17 patients). The frequency of HER2 abnormalities detected by the PRO Onc assay was similar in estrogen receptor-positive tumors (14 %) and triple-negative tumors (17 %).

Since the PRO Onc assay used an arbitrary cutoff level to define HER2 overexpression/activation, the actual levels found in the HER2-positive patients were examined. In eight patients, elevated HER2 levels were >2 times the cutoff level, while in 28 patients, the determined levels were <2 times above the arbitrary cutoff level.

Part 2 treatment results

In Part 2 of the study, 24 patients had HER2 abnormalities by PRO Onc assay, and 14 of these patients received HER2-targeted study treatment. The remaining ten patients did not meet all study eligibility criteria and were excluded (most frequently due to declining performance status on progression from their previous treatment). None of the 12 patients found to have HER2 overexpression/activation in Part 1 were treated in Part 2.

Table 3 contains a summary of the patient characteristics of the 14 patients who received treatment. Patients had received either one or two previous chemotherapy regimens for MBC. All ten patients with hormone receptor-positive tumors had received previous hormonal therapy. Ten patients had HER2 overexpression (median 3.55 CU; range 3.1–7.1), and seven patients had HER2 activation (median 0.9 CU; range 0.5–1.3). Three patients had both overexpression and activation.

Thirteen of the 14 patients received two cycles of therapy with trastuzumab+pertuzumab and were reevaluated. One patient was withdrawn from treatment because of

Table 2 Results of the PRO Onc assay ($N = 283$)

	Number of Patients (%)
CTCs identified	165 (57 %)
HER2 abnormalities detected	36 (22 %; 13 % of total population)
HER2 overexpression	19 (12 %)
1–2 × cutoff level 3.0 CU	16/19 (84 %)
>2 × cutoff level of 3.0 CU	3/19 (16 %)
HER2 activated without overexpression	17 (10 %)
1–2 × cutoff level 0.41 CU	12/17 (71 %)
>2 × cutoff level of 0.41 CU	5/17 (29 %)

Table 3 Characteristics of patients who received HER2-targeted treatment ($N = 14$)

Characteristic	Number of patients (%)
Median age, years, (range)	65 (47–83)
Race	
Caucasian	13 (93 %)
African American	1 (7 %)
ECOG Status	
0	9 (64 %)
1	4 (29 %)
2	1 (7 %)
Breast cancer subtype	
ER and/or PR positive	10 (71 %)
ER and PR negative	4 (29 %)
HER2 test used	
FISH	9 (64 %)
IHC	5 (36 %)
Median time from initial HER2 determination to date of PRO Onc Assay, months (range)	48 months (9–144 months)
Number of metastatic disease sites	
1	3 (21 %)
2	4 (29 %)
≥3	7 (50 %)
Prior chemotherapy	
Neoadjuvant/adjuvant chemotherapy	7 (50 %)
Chemotherapy for MBC	14 (100 %)
1 prior regimen	8 (57 %)
2 prior regimens	6 (43 %)
Previous anthracyclines	4 (29 %)
Previous taxanes	9 (64 %)
Prior hormonal therapy	10 (71 %)

rapid tumor progression prior to receiving two cycles of therapy.

When reevaluated after two cycles of therapy, 11 of the 13 patients had progressive disease and were removed from treatment. One patient had a partial response (measurable lung lesions), but developed progressive disease at the 12-week reevaluation. This patient was hormone receptor-positive, and had HER2 overexpression (4.4 CU) without activation. One patient had stable disease; because of inadequate symptom relief, the treating physician elected to add docetaxel to the trastuzumab/pertuzumab regimen. The patient subsequently had a partial response, but was removed from treatment one month later due to intercurrent illness (severe pneumonia).

No unexpected or unusual treatment-related toxicity was observed.

Discussion

Identification of critical molecular abnormalities within tumors has enabled the development of targeted agents, and has improved therapy. Molecular testing for specific abnormalities is now a standard part of clinical practice, since identification of specific patient subsets allows for appropriate targeted agents to be used. The development of HER2-targeted agents for the treatment of HER2-positive breast cancer has been one of the major successes for targeted therapy; HER2 status is now measured at the time of diagnosis, and subsequent HER2-targeted therapy in the adjuvant and metastatic settings results in major survival benefits.

In current practice, assays for specific molecular abnormalities are usually performed at the time of diagnosis, and are not repeated during the subsequent clinical course. However, as the understanding of tumor biology improves, several potential problems with this approach have been recognized. First, substantial molecular biologic diversity exists within most tumors; small biopsies from different portions of a tumor may show different molecular abnormalities [15]. Second, molecular abnormalities within tumors may change and evolve during the clinical course. In patients with breast cancer, HER2 determinations at diagnosis and at subsequent times in the disease course were discordant in 15–30 % of patients [10–12]. Finally, targeted therapy may induce specific molecular

abnormalities, resulting in resistance to the targeted therapy. For example, hormonal therapy for MBC results in activation of the PI3K/mTOR/Akt pathway, enabling the cancer to become hormone independent [16, 17].

All of these observations strongly suggest that periodic assays of critical molecular abnormalities during the clinical course of a cancer may be valuable in directing targeted therapy. However, the benefits of this approach have not yet been demonstrated, primarily due to practical difficulties in obtaining repeat biopsies. The recent availability of techniques to assay molecular abnormalities in tumors by isolating CTCs or by assaying for cell-free circulating tumor DNA may allow serial monitoring using blood specimens, without the need for multiple tumor biopsies [18, 19].

The PRO Onc assay is a multiplexed, proximity-based assay that can be performed on small tumor specimens obtained by fine-needle aspiration biopsy or on CTCs isolated from patients with metastatic cancer. The assay can detect abnormal expression of multiple tyrosine kinases, with sensitivity at the single-cell level [11]. In initial validation studies, HER2 status as determined by the PRO Onc assay was compared with IHC results in 227 breast cancers [12]. Results of HER2 status as determined by these two tests were concordant in 83 %, similar to previously determined concordance between IHC and FISH testing [5]. When immunoprecipitation western blot testing was added to IHC, the results of this combined testing were concordant with PRO Onc assay results in 96 % of tumors.

The PRO Onc assay was also tested on CTCs isolated from 27 patients with metastatic breast cancer [12]. Of the 17 patients with HER2-negative breast cancer (as defined by IHC), 7 had HER2 amplification and activation detected in their CTCs. In ten patients who were initially HER2-positive, six retained CTC HER2-positivity, while four were HER2-negative. Similar levels of discordance between initial HER2 determination (primary tumor) and subsequent assays of CTCs or cell-free DNA have been reported with other methods [9, 10, 20]. The possibility that patients who had HER2-positive CTCs could benefit from HER2-targeted therapy provided a rationale to further evaluate these findings.

In this study, we used the PRO Onc assay to test blood specimens from 283 women with metastatic HER2-negative breast cancer. The median interval since previous determination of HER2 status was 36 months (range <1–245 months), and all women had received at least one chemotherapy regimen in the interim. Circulating tumor cells were identified in 57 % of the specimens, an incidence similar to that which has been previously reported with this assay method [21, 22]. Overall, 36 of 160 specimens (23 %) with CTCs identified had either HER2 overexpression or activation; the much lower incidence in the larger group assayed in Part 2 (24 of 129; 19 %) versus Part 1 (12 of 31; 39 %) is unexplained. Therefore, our study confirms the previous findings using the PRO Onc assay in HER2-negative patients, and demonstrates HER2 abnormalities by PRO Onc assay in a substantial minority of patients who are HER2-negative by standard tests.

Unfortunately, we were unable to identify a group of patients who benefited from HER2-targeted therapy. In Part 2 of our study, 14 patients received HER2-targeted therapy, and all but two patients progressed within the first 6 weeks. One patient had a partial response (duration 12 weeks), and the single patient with stable disease at first reevaluation had docetaxel added but was removed from study at week 13.

Several possible explanations for the negative results of this study should be considered. First, the relatively small number of patients who actually received HER2-targeted therapy may have been insufficient to rule out activity. In the initial clinical experience with trastuzumab, performed in a similar heavily treated patient population, the single-agent response rate was only 15 % [14]. However, an additional 29 % of patients had stable disease, some of which persisted for >6 months. More recently, the combination of trastuzumab and pertuzumab produced an objective response rate of 24 % and a clinical benefit rate of 50 % in a group of less heavily pretreated patients with metastatic HER2-positive breast cancer [23]. In contrast, the majority of our patients progressed rapidly during the first 6 weeks of treatment, and all had progressed by the 12-week reevaluation. Although these results do not rule out the possibility that an occasional patient may respond, our study do not suggest a clinical role for the PRO Onc assay in screening patients with metastatic HER2-negative breast cancer.

A second possibility for the negative results may be related to the PRO Onc assay itself. The assay was validated in a relatively small number of CTC specimens from patients with metastatic breast cancer. The definitions of HER2 overexpression and activation were arbitrary, based on signals obtained from specimens of normal volunteers. However, no activity of HER2-targeted therapy was seen in patients with the highest HER2 levels detected (>2 times the cutoff level).

A more troubling possible explanation for our negative findings is that tumor abnormalities identified in the blood (from small numbers of CTCs or small DNA fragments) do not accurately reflect the molecular characteristics of the tumor as a whole, and do not correlate well with response to targeted treatment. However, increasingly refined methods are being developed to measure various tumor molecular abnormalities from blood specimens, and intensive efforts to evaluate their clinical utility are ongoing. Recently, detection of T790M mutations in the blood

or urine in patients with advanced NSCLC was found to be a strong predictor of response to the EGFR inhibitor rociletinib [24]. In breast cancer, anecdotal reports have documented responses to HER2-targeted therapy in HER2-negative patients found to have HER2-positive CTCs or circulating HER2 fragments [25]. In addition, an ongoing randomized phase III trial (DETECT III) is comparing the efficacy of physicians' choice chemotherapy±lapatinib in patients with HER2-negative metastatic breast cancer and HER2-positive CTCs [26]. Although data are just beginning to accumulate, it seems likely that the clinical utility of blood "biopsies" will be assay-specific as well as tumor-specific.

In summary, the PRO Onc assay identified HER2 overexpression or activation in 23 % of HER2-negative women who had MBC and detectable CTCs. However, HER2-targeted therapy was not effective in a group of 14 women identified in this manner. These study results do not allow a determination as to whether the lack of treatment efficacy was related to the selection of inappropriate patients (i.e., assay-related) or whether CTC HER2-positivity lacks predictive power for response to HER2-targeted treatment. At present, HER2 status should be determined from a tumor biopsy specimen, using established FISH or IHC criteria.

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Compliance with ethical standards

Conflict of interest JDH received funding to his institution for the study from Genentech and Prometheus. All the other authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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