HER-2 gene amplification can be acquired as breast cancer progresses

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Amplification and overexpression of the *HER-2* oncogene in breast cancer is felt to be stable over the course of disease and concordant between primary tumor and metastases. Therefore, patients with *HER-2*-negative primary tumors rarely will receive anti-Her-2 antibody (trastuzumab, Herceptin) therapy. A very sensitive blood test was used to capture circulating tumor cells (CTCs) and evaluate their *HER-2* gene status by fluorescence *in situ* hybridization. The *HER-2* status of the primary tumor and corresponding CTCs in 31 patients showed 97% agreement, with no false positives. In 10 patients with *HER-2*-positive tumors, the *HER-2*/chromosome enumerator probe 17 ratio in each tumor was about twice that of the corresponding CTCs (mean 6.64 ± 2.72 vs. 2.8 ± 0.6). Hence, the ratio of the CTCs is a reliable surrogate marker for the expected high ratio in the primary tumor. Her-2 protein expression of 10 CTCs was sufficient to make a definitive diagnosis of the *HER-2* gene status of the whole population of CTCs in 19 patients with recurrent breast cancer. Nine of 24 breast cancer patients whose primary tumor was *HER-2*-negative each acquired *HER-2* gene amplification in their CTCs during cancer progression, i.e., 37.5% (95% confidence interval of 18.8–59.4%). Four of the 9 patients were treated with Herceptin-containing therapy. One had a complete response and 2 had a partial response.

Considerable clinical data demonstrate that Her-2/neu (Her-2) overexpression, usually attributable to *HER-2* gene amplification, occurs in ≈20–25% of breast cancer patients and is associated with a poor prognosis (1). Cancer cells that overexpress Her-2 are often resistant to many cytotoxic drugs and radiotherapy (2). However, a humanized monoclonal antibody, trastuzumab (Herceptin, Genentech) can effectively treat tumors with *HER-2* gene amplification in 25% of patients as monotherapy (3) and 50% when given with taxane or other chemotherapy (4). Remission can last 1–2 years until the tumor cells become resistant (2, 4).

The diagnosis of Her-2 overexpression and/or *HER-2* gene amplification is made on the primary tumor. Comparison of the immunohistochemical methods to determine overexpression and fluorescence *in situ* hybridization (FISH) to determine gene amplification has indicated that the latter is more accurate and more predictive of a favorable response (2). The 70–75% of patients who do not have *HER-2* gene amplification in their primary tumors are rarely diagnosed with such amplification at a later date because biopsies are done infrequently and usually not examined for *HER-2* status. Therefore, if *HER-2* gene amplification can be acquired, it is important to develop a safe and definitive method for making this diagnosis so that such patients can receive optimal treatment.

We have developed a sensitive blood test to detect and characterize circulating tumor cells (CTCs) (5). CTCs can be detected in most primary tumors and in virtually all patients with a recurrence of breast cancer either not yet on treatment or between therapeutic regimens, or when patients are chemorefractory and the tumor is progressing (5, 6). Signals from FISH examination of CTCs can be quantified precisely because the CTCs do not overlap and are flattened against the slide. The result is that *HER-2* gene amplification can be accurately measured in individual cells. This method was used to determine whether there is concordance between the pathologist's analysis of *HER-2* gene status in primary tumors and the corresponding CTCs, whether *HER-2* gene amplification can be acquired with tumor progression, and, if so, whether such patients can respond to targeted therapy.

Materials and Methods

Patient Selection and Data Recording. Pertinent personal and clinical data were recorded for every patient with carcinoma who participated in this study. All specimens were obtained with informed consent and collected by using protocols approved by the Institutional Review Board at the University of Texas Southwestern Medical Center.

Collection of Samples. Thirty milliliters of blood was drawn in 10-ml Vacutainer tubes (BD Biosciences) containing EDTA and processed within 4 h of collection.

Cell Lines. Carcinoma cell lines SKBr3, Colo 205, PC3, and BT474, used as control cells and for testing of reagents, were grown in RPMI medium 1640 plus 10% FCS.

Ferrofluids for CTC Enrichment. CTCs were immunomagnetically enriched with ferrofluids (7) conjugated to antibody against epithelial cell adhesion molecule (specific for epithelial cells) (8, 9). This antiepithelial cell adhesion molecule antibody GA73.3 (provided by D.H.) was bound to ferrofluids by Immunicon Corp.

Isolation of CTCs. Blood was processed as described in ref. 5, except that 2 mM EDTA was added to the wash buffer and the cells were not permeabilized. Samples were washed, the supernatant was aspirated and resuspended in 100 μl of PBS per 5 ml of blood, and 100 μl was placed on each slide and air-dried at 37°C. Slides were stored at −80°C.

Antibodies. (*i*) Monoclonal mouse anti-pan-cytokeratin clone C11-FITC (Sigma); (*ii*) monoclonal mouse anti-CD45 (clone 9.4 from

Abbreviations: CEP, chromosome enumerator probe; CK, cytokeratin; CTC, circulating tumor cell; FISH, fluorescence *in situ* hybridization; IF, immunofluorescence; mam, mammaglobin.

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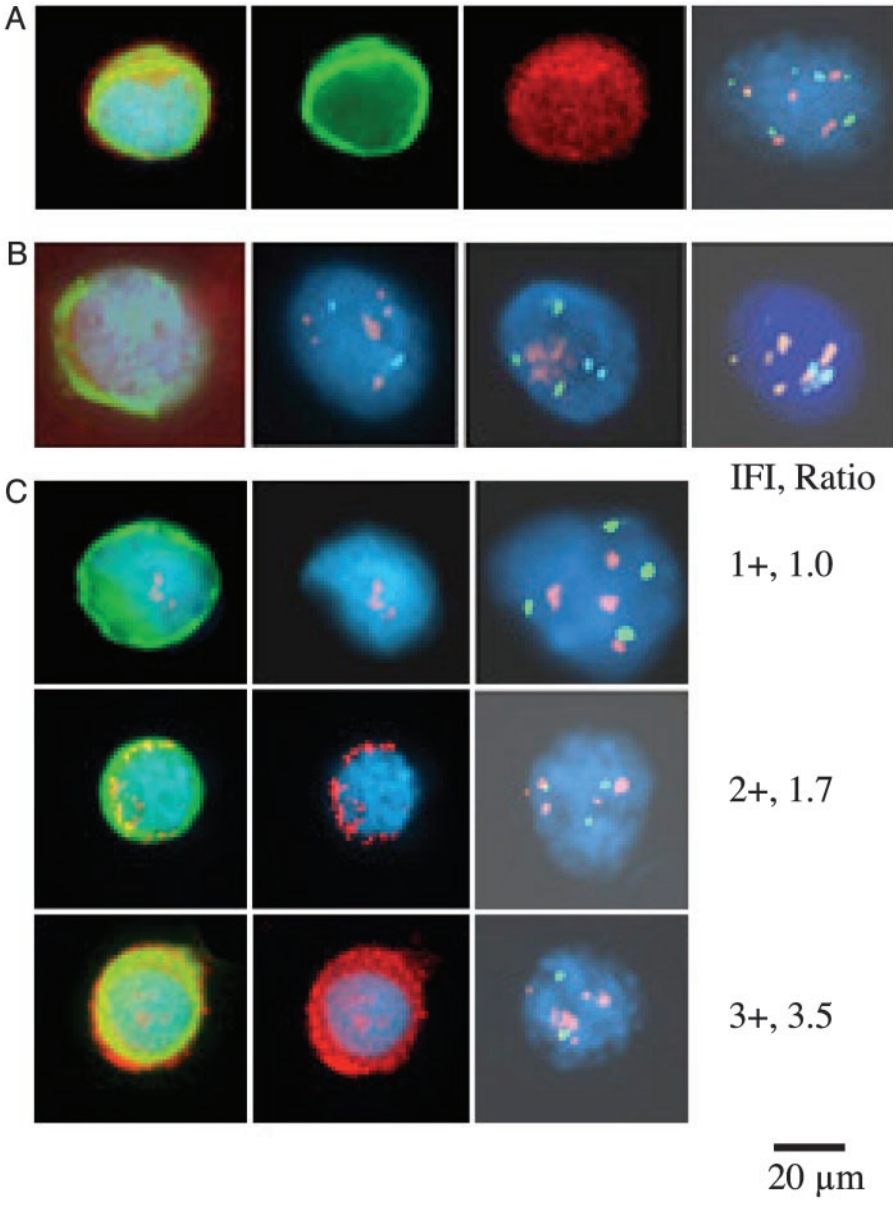

Fig. 1. Fluorescent microscopic images of cytomorphology, immunophenotype, and FISH evaluation of CTCs of patients with metastatic breast cancer. (*A*) Decomposing the immunophenotype and displaying aneuploidy of a CTC from a metastatic breast patient. Single-band filters were used to block out the fluorescence of one or more fluorochromes used to stain for CTCs. Horizontally, the photos show the same cell stained for the combination of CK (green), mam (red), and nucleic acid (blue); CK only; mam only; and FISH analysis. For FISH, CEP 1 (SpectrumOrange), CEP 8 (SpectrumAqua), and CEP 17 (SpectrumGreen) were used (counts were 5, 2, and 5, respectively). The photos are taken in only one *Z*-plane, whereas the microscopist can focus on the entire range of *Z*-planes; therefore, spots that are not seen or overlap in the single *Z*-plane of the photo can be distinguished by microscopy. (*B*) Sequential genotyping of a CTC isolated from blood of a metastatic breast cancer patient. First photo, epithelial cell detected by an anti-CK-antibody (green) and nucleic acid (blue). Second photo, same cell hybridized with a locus-specific probe for c-myc (orange) and CEP 8 (aqua) (7, 3). Third photo, same cell hybridized with Her-2 (orange), CEP 10 (green), and CEP 17 (aqua) (6, 2, and 3). Fourth photo, same cell hybridized with CEP 1 (orange), CEP 8 (aqua), and CEP 17 (green) (6, 3, and 2). (*C*) Comparison of the intensity of Her-2 IF staining with *HER-2* gene amplification of CTCs from metastatic breast cancer. Three rows representing Her-2 IF staining intensity of a single cell of 1+ (first row), 2+ (second row) and 3+ (third row) are shown. Photo 1 of each row shows staining for the combination of CK, Her-2, and nucleic acid. Photo 2 shows IF staining for Her-2 and nucleic acid. Photo 3 shows FISH results for *HER-2* (SpectrumOrange) and CEP 17 (SpectrumGreen). Her-2 IF staining intensity (IFI) and *HER-2*/CEP 17 ratio (Ratio) for each cell are shown.

ATCC) conjugated in our laboratory to Alexa Fluor 546 (Molecular Probes); (*iii*) anti-Her2 (Her 81) raised against the extracellular domain of Her-2 (provided by E.V. and conjugated to Alexa Fluor 594); and (*iv*) rabbit polyclonal anti-mammaglobin (anti-mam) (provided by T.F.) $F(ab')_2$ fragment conjugated to Alexa Fluor 594 in our lab.

Immunofluorescence (IF) Staining. (*i*) IF staining was carried out as described in ref. 5. Blood slides from healthy individuals of similar ages served as negative controls, and SKBr3 cells (a Her-2-overexpressing breast carcinoma cell line) served as positive controls.

(*ii*) Screening for CTCs was performed as described in ref. 5 to locate cytokeratin $(CK)^+$ $CD45^-$ cells. Her-2 IF (0–3+) was evaluated at this time. The location of each candidate cell was recorded and stored. Slides from normal donors and patients were coded so that investigators were "blinded."

Multicolor FISH. Pretreatment and denaturation of slides are described in detail in ref. 5. Chromosome enumerator probes (CEPs) 1 (SpectrumOrange), 8 (SpectrumAqua) 10 and 17 (SpectrumGreen), and Her-2 and locus-specific probe for c-myc (SpectrumOrange) were provided by Vysis (Downers Grove, IL). Hybridization and posthybridization washes were performed according to manufacturer's instructions. Slides were counterstained and prepared with mounting medium containing 4′,6-diamidino-2-phenylindole. Leukocytes from patients served as controls.

Multicolor FISH Evaluation. Hybridized cells were relocated with the same fluorescence microscope used for scanning. Hybridization signals in recorded cells were counted separately for each probe through the appropriate single-pass filter. For each relocated cell, an image was recorded. The leukocytes on the patients' slides were used as control cells for hybridization efficiency as described in ref. 5.

Statistical Analysis. To compare the concordance of the *HER-2* status between tumor and CTCs, we performed exact statistical analysis with statistical software for exact nonparametric interfer-

Table 1. *HER-2* status in primary tumor and CTCs

Patient no.	Pathology TNM	Pathology Stage	Blood test data: Time after pathology results, months	No. of CTCs	Ratio *HER-2*/CEP 17	Treatment within 1 month prior to blood test
Concordant: pathology and CTCs both positive						
1*	T4N2MX	IIIB	0	12	4.0	None
2*	—	—	0	23	2.5	None
3*	T1aN1M0	II	0	9	2.6	None
4*	T3N1M0	IIIA	0	50	3.4	None
5*	T3N1MX	IIIA	0	11	2.6	None
6*	T2NXMX	—	0	11	3.4	None
7*	—	—	0	4	2.0	None
8*	T4N1M0	IIIB	0	9	2.7	None
9†	T2N0M0	IIA	50	38	2.2	None
10†	T3N1MX	IIIA	5	7	2.1	None
11†	T1N0M0	I	0	25	2.3	None
Concordant: pathology and CTCs both negative						
12*	T4N2M1	IV	0	14	1.2	Chemo
13*	T4M1	IV	0	7	1.3	None
14*	T3NXMX	—	0	15	1.5	None
15†	—	—	26	26	1.2	Chemo
16†	T2N1M0	IIB	29	16	1.2	None
17†	T1N0M0	I	126	37	1.2	None
18*	T1aN0M0	I	37	66	1.3	Chemo and hormonal
19*	T4N3M1	IV	120	54	1.5	None
20*	T4N0M1	IV	60	11	1.2	Chemo and hormonal
21†	T3N1M0	IIIA	43	5	1.8	None
22†	T1cN0M0	I	37	5	1.5	None
23†	T2NXM1	IV	28	61	1.3	Chemo
24†	T2N1M1	IV	168	11	1.7	Chemo and hormonal
25†	T3N1MX	IIIA	60	15	1.2	None
26†	T1N1M0	IIA	24	14	1.6	Chemo and hormonal
27†	T1N1M0	IIA	17	50	1.1	Chemo and hormonal
28†	T1N1M0	IIA	—	5	1.0	None
29†	T2N1M0	IIB	84	60	1.5	None
Nonconcordant: pathology positive, CTCs negative						
30*	T3N1M0	IIIA	0.5	60	1.6	Herceptin
31*	T1N0M0	I	0	53	1.3	None
32†	T3N1M0	IIIA	18	12	1.1	Herceptin, chemo, and hormonal
33*	T2N1M0	IIB	0	60	1.6	Chemo

TNM, tumor–node–metastasis staging. —, information not available. Chemo, chemotherapy. Twenty-five patients had blood samples of ≤10 ml. Nine patients had blood samples of 15–20 ml.
*Patients were evaluated by our blood test at primary tumor removal.
†Patients were evaluated by our blood test at recurrence.

ence (STATXACT 6.0, Cytel Software, Cambridge, MA) by using binomial distribution with concordance categorized as a success. The same analysis was performed to determine sufficiency of 10 CTCs for diagnosis.

Results

Criteria for CTCs. Our criteria for identification of a CTC include cytomorphology, immunophenotype, and aneusomy. Fig. 1*A* shows a classical CTC: large round cell, high nuclear-to-cytoplasmic ratio, staining of cell periphery with anti-CK, anti-mam staining of both periphery and cytoplasm of cell, and no staining with anti-CD45, a WBC marker. Fig. 1*B* shows anti-CK staining and aneusomy in a CTC probed three times by FISH. Our classification of a circulating cell as a CTC is unambiguous. In the present studies, anti-Her-2 was used instead of anti-mam, and CEP 17 and *HER-2* were the DNA probes.

Concordance. We determined whether the results of the blood test were concordant with those obtained from the primary tumor, the current "gold standard." Table 1 summarizes these studies. CTCs were isolated and examined for *HER-2* gene status (Table 1 and Fig. 1 *B* and *C*). Conventional analysis for *HER-2* gene amplification was performed by the pathology laboratory on 33 primary tumors (15 tumors were *HER-2* gene amplified and 18 were nonamplified) to determine concordance of their *HER-2* status with their corresponding CTCs. Of the nonconcordant results, 3 patients had *HER-2*-amplified tumors with *HER-2*-nonamplified CTCs. However, these patients had previously been treated with Herceptin (nos. 30 and 32) or with chemotherapy (no. 33) before the blood sample was obtained. Therefore, it was not possible to score concordance in these patients. Our initial results, therefore, indicated 97% (95% confidence interval of 84–100%) concordance. There was one patient (no. 31) with Her-2 overexpression in which we found no *HER-2* gene amplification. Such nonconcordance has no major clinical implications because the patient will be classified as *HER-2* positive.

Number of CTCs Needed to Determine *HER-2* Gene Status. Because of the small number of patients and the wide range of CTCs per 10 ml in each patient, we arbitrarily chose subsets of 10 consecutive CTCs (bins) per patient to determine whether each bin in a patient would reflect the gene status of the patient. The CTCs of 30 patients [18 were *HER-2* gene nonamplified (930 CTCs) and 12 were *HER-2* gene amplified (480 CTCs)], all of whom had 20–60 CTCs counted, were divided into "bins" each containing 10 consecutive CTCs. The results indicated that 139 of 141 bins were concordant with the

Table 2. Acquisition of *HER-2* amplification with tumor progression/treatment

Patient no.	Pathology: TNM	Pathology: Stage	Blood test data: Time between recurrence and blood tests, months	Blood test data: No. of CTCs	Blood test data: Ratio *HER-2*/CEP 17	Adjuvant treatment	Postrecurrence treatment
34	T3N1M0	IIIA	24	102	2.4	Four cycles of Adriamycin and Cytoxan, followed by radiation to chest wall and 5 years of tamoxifen	Aromasin and Femara, no chemo
35	T4bN0M0	IIIB	15	42	2.3	Four cycles of Adriamycin and Cytoxan	Tamoxifen
36	T3N1M0	IIIA	—	60	2.4	Four cycles of Adriamycin and Cytoxan, treatment with Taxol followed by radiation	None
37	—	—	12	26	2.2	Four cycles of high-dose Adriamycin and Cytoxan, followed by autologous stem cell rescue and 3 years of tamoxifen	Aredia, Lupron, Femara, Zometa, Navelbine, and Flaslodex
38	T0N1M0	II	96	20	2.6	Radiation	Tamoxifen, Arimidex, Megace, Gemzar, Faslodex
39*	—	—	60	16	2.0	Six cycles of FAC followed by 3 years of tamoxifen	Megace, Taxotere, Cytoxan × 6, high dose marrow transplant, Arimidex, Aredia, Femara, Xeloda, AVI-007, Zometa, and Doxil
40	T3N1M0	IIIA	24	50	2.5	Six cycles CAF, followed by radiation and 4 years of tamoxifen	Lupron and Xeloda
41	T4N2M0	IIIB	6	7	2.5	Four cycles of Adriamycin and Cytoxan, Taxotere, and Xeloda followed by radiation	Vinorelbine, Zometa
42	T2N1M1	IV	16	6	2.7	Four cycles of Adriamycin and Cytoxan	Adriamycin

HER-2: Primary tumor negative, CTC positive. —, information not available; FAC, fluorouracil, Adriamycin, and cyclophosphamide; CAF, cyclophosphamide, Adriamycin, and 5-fluorouracil.
*Tumors were evaluated by FISH for these patients, except no. 39.

overall *HER-2* gene status of the patients (95% confidence interval of 95–100% for concordance). Neither of the 2 nonconcordant bins, which were from different patients, affected the gene status of the patient. Thus, 10 CTCs may be sufficient to determine the *HER-2* status of most patients. However, that does not exclude the possibility that fewer CTCs with a high ratio may be sufficient to indicate that a patient is a candidate for trastuzumab-containing therapy. The number of CTCs that are sufficient for diagnosis must be reinvestigated with more patients. The sensitivity and specificity of different numbers of CTCs for correctly calculating the *HER-2* status also must be determined. Receiver operating characteristic curves should be plotted and the number of CTCs providing the best sensitivity and specificity should be selected as the most appropriate threshold.

Acquisition of *HER-2* Gene Amplification. We have studied 24 patients whose primary tumor was reported as *HER-2* negative who developed a recurrence. We obtained a blood sample from each patient before treatment was initiated, between chemotherapeutic regimens, or when the patient had become chemorefractory with progressive tumor growth. Of these 24 patients, 9 developed *HER-2* gene amplification in their CTCs as shown in Table 2.

Relationship Between *HER-2* Gene Amplification in CTCs and Corresponding Primary Tumor. Patients who acquired *HER-2* gene amplification in their CTCs had ratios of 2.0–2.7. These are relatively low ratios and raise concerns that such patients may not respond to trastuzumab-containing therapy. To address this concern, we compared the ratio between each *HER-2*-positive primary tumor and its corresponding CTCs. Table 3 shows 10 primary tumor tissues evaluated by FISH for *HER-2* gene status in our medical school pathology laboratory. *HER-2* gene ratios in the primary tumor were an average of 2.44-fold higher (95% confidence interval of 1.91–2.98) than the corresponding CTCs. This result indicates that the comparatively low ratio of *HER-2* gene amplification in CTCs is a consistently reliable surrogate marker for the higher gene amplification of the corresponding tumor. The mechanism(s) responsible for this difference is not known.

Treatment of Patients Who Acquired *HER-2* Gene Amplification with Tumor Progression. Clinicians assessed clinical response by using the Response Evaluation Criteria in Solid Tumors (RECIST) (10). In addition, biochemical response was monitored for factors including biomarkers for breast cancer (CA 27.29, etc.) and surrogate markers of response or progression. However, they were only used in conjunction with clinical evaluation.

The first patient (no. 36) entered the hospital in liver and renal failure and was moribund. As shown in Table 4, the CTCs that were *HER-2* amplified were preferentially eliminated, indicating a role for the Herceptin and the cisplatin. She had a remarkably rapid remission that lasted >1 year with complete disappearance of tumor. However, her CA 27.29 is now rising, although she remains

Table 3. Ratio of *HER-2* gene amplification of primary tumor and corresponding CTCs

Patient no.	Ratio *HER-2*/CEP 17: Tumor	Ratio *HER-2*/CEP 17: CTCs
1	13.60	4.0
2	9.00	2.5
3	6.46	2.5
4	5.01	3.4
5	3.47	2.6
6	6.00	3.4
7	7.16	2.0
8	5.01	2.7
10	5.67	2.1
11	5.00	2.3
Mean ± SD	6.64 ± 2.86	2.8 ± 0.7

Table 4. Treatment of patients who acquired *HER-2* gene amplification

Patient no.	Time after treatment, days	No. CTCs analyzed	No. CTCs/ 5 ml of blood	Ratio *HER-2*/CER 17	Treatment	Clinical response*	Laboratory†
36	0	60	2,400	2.4	Cisplatin and Herceptin	Complete response‡	CA 27.29 = 980 units/ml Bilirubin = 17 mg/dl
	5	60	500	1.6	Navelbine and Herceptin		
	13	33	40	1.5			
	309	5	2	1.5	Herceptin		CA 27.29 = 25.8 units/ml Bilirubin = 0.7 mg/dl
	364	6	2	1.3			
	477	4	1	1.6	Navelbine and Herceptin		CA 27.29 = 133.4 units/ml Bilirubin = 1.3 mg/dl
37	0	26	10	2.2	Femara, Flaslodex,	Partial response	
	159	22	60	1.6	Navelbine, and Herceptin	Disappearance of nodes and gastric cancer	
23	0	61§	70	1.3	Navelbine and Herceptin	Partial response¶	30% decrease of CA 27.29
	27	40	50	1.0		Decrease in size of skin lesions	
39	0	16	30	2.0	Zometa, Taxotere, Doxil,	Disease progression¶	
	103	13	15	1.5	and Herceptin		

*Clinical response according to RECIST criteria as reported by the patients' oncologist.
†Normal CA 27.29 is 0.0–38.0 units/ml. Normal bilirubin is 0.2–1.3.
‡Initial radiographic studies showed a huge liver almost completely replaced by tumor, numerous tumor nodules in the lung, and pleural effusions. By day 364, there were no active metastatic lesions in the liver and chest x-ray was normal.
§20% of the CTCs were *HER-2*$^+$ (ratio = 2.2) before treatment and only 2.5% after treatment.
¶Died of brain metastases.

asymptomatic. Two additional patients (nos. 23 and 37) had documented partial responses and one patient had no response (no. 39). One of these patients (no. 23) had CTCs that showed an average ratio of only 1.3, but 20% of her CTCs indicated *HER-2* gene amplification (ratio of 2.2). The patient was chemorefractory and displayed tumor progression. Herceptin was added to her chemotherapeutic regimen. The CTCs with *HER-2* gene amplification were virtually eliminated and, unexpectedly, there was a partial response.

Assay to Determine Her-2 Overexpression. In pathology laboratories, the evaluation of *HER-2* status begins with immunohistochemical analysis for expression of Her-2 protein. Immunohistochemical is simple and inexpensive compared with FISH. For future experiments using CTCs, a similar sequence of assays would be desirable. Because immunohistochemical analysis inhibits subsequent analysis by FISH on the same slide, it was necessary to develop an IF assay. By using a high-affinity murine antihuman Her-2 protein (Her-81)-Alexa Fluor 594 with a nucleic acid dye (4′,6-diamidino-2-phenylindole), anti-CK-FITC, and anti-CD45-Alexa Fluor 546, we stained Her-2 protein on CTCs from patients with metastatic breast cancer. Three different densities of Her-2 protein were readily distinguishable (Fig. 1*C*). Twenty to 60 CTCs from 19 patients were scored as 0–3+ Her-2 expression before FISH was performed. By using subsets of 10 consecutive cells (bins), the average of each bin for Her-2 expression and *HER-2* amplification was calculated. Of the 14 patients who had 0–2+ expression (33 bins), none were gene amplified. All 5 of the 3+ patients (9 bins) were gene amplified (2.0–4.0). There was concordance of all bins within each patient. Based on our analysis of 42 bins, we conclude that Her-2 expression predicts *HER-2* gene amplification with high probability (95% confidence interval of 93–100%).

Discussion

The *HER-2* status of the primary tumor has been the gold standard for many years. It is assumed that if the primary tumor of a breast cancer patient is *HER-2* negative, she will not acquire *HER-2* gene amplification (*HER-2* copy number per CEP 17 copy number is ≥2.0) as her cancer progresses. This conclusion was reached because of reports indicating concordance between the *HER-2* status of the primary tumor and metastases in the same patient (11–13). In most of these papers, the metastases were obtained at the same time as the primary tumor; hence, concordance would be expected. However, there are other reports of concordance of *HER-2* status in which metastases were obtained asynchronously to the primary tumor (14, 15). These reports did not indicate whether the patients were treated intensively with chemotherapy, radiotherapy, or hormonal therapy before the biopsy was taken. Indeed, many of these biopsies may have been obtained at the time of recurrence. In our studies, virtually all of the patients who acquired *HER-2* gene amplification in their CTCs either had been treated intensively with chemotherapy or radiation therapy or were far advanced and, in several cases, moribund. We postulate that these selective pressures are essential for the few *HER-2*$^+$ variants, either already present in the *HER-2* negative primary tumor or acquired by mutation, to "overtake" the non-*HER-2*-amplified tumor cells. Several papers strongly support our conclusion in that a significant percentage of patients with Her-2-negative primary tumors develop high concentrations of the extracellular portion of Her-2 in their serum with tumor progression (16–19). Finally, Walker *et al.* (20), using a method for measuring the number of Her-2 molecules per CTC, followed 19 patients with recurrent breast cancer for many months with repeated blood samples. They observed that 3 patients who had nonoverexpressing CTCs suddenly developed a relapse clinically preceded by a rise in CTCs with very high levels of Her-2 per CTC. In general, the above reports support the possibility that *HER-2* gene amplification can be acquired during progression of the cancer. However, since the dogma that the primary tumor "tells all" was established, there has been little interest to study further whether *HER-2* gene amplification can be acquired as cancer progresses. Because it is well documented that the original genetically unstable tumor clone continues to mutate at a rapid rate (21) and is constantly giving rise to variants that are resistant to the particular therapeutic regimen used (2), we challenge the decision that the primary tumor should be the gold standard for making treatment decisions at a later date.

The development of targeted therapy represents a shift in paradigm from the unrealistic attempt to kill all cancer cells with high-dose chemotherapy, to attempting to control residual cancer with targeted therapy after removal of the primary tumor. Trastu-

zumab is one example of several noncytotoxic drugs that can induce regression of tumor. Moreover, there is a major effort to develop additional molecules that can control residual tumor. Hence, in the future, the oncologist may have 5–10 such targeting molecules in his or her armamentarium to treat patients with recurrent cancer.

A major obstacle in treating any tumor is that the tumor cells are constantly changing and, at present, the oncologist does not know what changes have taken place. The small percentage of biopsies that are performed are infrequently investigated for Her-2 overexpression, and repeated biopsies cannot be performed to evaluate the additional changes that are likely to accompany cancer progression. Also, metastases, which can be monoclonal or pauciclonal, can differ with regard to *HER-2* status (20). In contrast, obtaining a blood sample is safe and can be performed repeatedly. Analysis can be automated and yield more valid *HER-2* gene ratios than the pathological diagnosis.

The extent of acquired *HER-2* gene amplification in CTCs was low, usually 2.0–3.0. However, comparison of amplification between *HER-2* gene amplified primary tumors (Table 3) and CTCs indicates that each tumor had ≈2–3 times the amplification of the CTCs in all of the patients studied. Therefore, the CTCs should provide a reliable surrogate ratio for the amplification of the *HER-2* gene in metastases as well. A likely explanation for this consistent discrepancy is that the subset of tumor cells at the growing edge of the tumor examined by the pathologist is a different subset from the one shedding CTCs, which may come from tumor adjacent to blood vessels. A more rapid rate of apoptosis of *HER-2*-amplified CTCs or rapid elimination because of overexpression of Her-2 itself or another overexpressed protein encoded in the same amplicon cannot be excluded.

Responses of several terminal patients who had acquired *HER-2* gene amplification in their total population of CTCs (Table 4), or in the one patient in which only a subset had *HER-2* gene amplification, were encouraging. In particular, patient 36 probably would have died from hepatic and renal insufficiency within 48 h had she not been treated with Herceptin and cisplatin. The rapidity of her remission with preferential killing of *HER-2* gene amplified cells within 5 days accompanied by a symptom-free period of 18 months is impressive.

A particularly important advantage for the CTC assay is that the gene status of individual cells can be ascertained. Thus, one patient (no. 23) who had only a subset of CTCs that were amplified, had a favorable clinical response when Herceptin was added to her chemotherapy. In the future, when many targeting drugs are available, a scenario can be visualized in which different portions of a progressing tumor have different genetic changes that can be targeted. Different subsets of cells could have amplification of different genes such as *HER-2*, *EGFR*, *uPAR*, etc., but conventional pathological examination could conclude that there was no amplification in the entire population for any of the genes. In contrast, the examination of individual CTCs could measure the number of CTCs in a subset that is amplified for each gene. The results might indicate that a combination of targeted drugs should affect almost all of the CTCs, and, therefore, the appropriate targeted drugs should be given in combination.

Another advantage of examining individual cells is that the IF intensity of an anti-Her-2 fluorochrome conjugate can be compared in the same cell to the precise *HER-2*/CEP 17 ratio (Fig. 1*C*). Therefore, the correlation between intensity of staining and gene amplification can be readily studied because each patient usually has many cells. The data presented in Fig. 1*C* and expanded in the *Discussion* indicate that Her-2 expression in CTCs as measured by using IF predicted *HER-2* gene amplification in all 19 patients. These preliminary findings further emphasize the precision of determining gene copy numbers and Her-2 expression in CTCs.

This study with its small number of patients represents a proof-of-principle report. Obviously, much more data involving many patients must be obtained to conclude that patients whose CTCs acquire *HER-2* gene amplification should be treated with trastuzumab-containing therapy as a standard procedure. In particular, to reach that conclusion, two additional studies must be completed: (*i*) Biopsy of metastatic tumor from patients who have acquired *HER-2* gene amplification in their CTCs indicates the usual high *HER-2* gene amplification ratio as seen in the primary tumor. (*ii*) A portion of such patients should respond to either monotherapy with trastuzumab or addition of trastuzumab to a chemotherapeutic agent that has lost its effectiveness. However, there is already sufficient data to warrant further study of acquisition of *HER-2* gene amplification by using CTCs because if they can reflect the status of the recurrent tumor, they would represent a safe "real-time" biopsy to detect genetic changes in general as cancer progresses.

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