ORIGINAL ARTICLE

Analysis of Circulating Tumor DNA to Monitor Metastatic Breast Cancer

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ABSTRACT

BACKGROUND

The management of metastatic breast cancer requires monitoring of the tumor burden to determine the response to treatment, and improved biomarkers are needed. Biomarkers such as cancer antigen 15-3 (CA 15-3) and circulating tumor cells have been widely studied. However, circulating cell-free DNA carrying tumor-specific alterations (circulating tumor DNA) has not been extensively investigated or compared with other circulating biomarkers in breast cancer.

METHODS

We compared the radiographic imaging of tumors with the assay of circulating tumor DNA, CA 15-3, and circulating tumor cells in 30 women with metastatic breast cancer who were receiving systemic therapy. We used targeted or whole-genome sequencing to identify somatic genomic alterations and designed personalized assays to quantify circulating tumor DNA in serially collected plasma specimens. CA 15-3 levels and numbers of circulating tumor cells were measured at identical time points.

RESULTS

Circulating tumor DNA was successfully detected in 29 of the 30 women (97%) in whom somatic genomic alterations were identified; CA 15-3 and circulating tumor cells were detected in 21 of 27 women (78%) and 26 of 30 women (87%), respectively. Circulating tumor DNA levels showed a greater dynamic range, and greater correlation with changes in tumor burden, than did CA 15-3 or circulating tumor cells. Among the measures tested, circulating tumor DNA provided the earliest measure of treatment response in 10 of 19 women (53%).

CONCLUSIONS

This proof-of-concept analysis showed that circulating tumor DNA is an informative, inherently specific, and highly sensitive biomarker of metastatic breast cancer. (Funded by Cancer Research UK and others.)

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Breast cancer is the most common cancer and the leading cause of cancer-related death in women worldwide.[1] Metastatic breast cancer remains an incurable disease but is treatable by means of serial administration of endocrine, cytotoxic, or biologic therapies. The monitoring of treatment response is essential to avoid continuing ineffective therapies, to prevent unnecessary side effects, and to determine the benefit of new therapeutics. Treatment response is generally assessed with the use of serial imaging, but radiographic measurements often fail to detect changes in tumor burden. Therefore, there is an urgent need for biomarkers that measure tumor burden with high sensitivity and specificity.

Cancer antigen 15-3 (CA 15-3) is a serum biomarker that is clinically useful in some patients with metastatic breast cancer but has a sensitivity of only 60 to 70%.[2-4] The enumeration of circulating tumor cells has emerged as a promising biomarker. Although there are numerous methods to detect circulating tumor cells in the research setting,[5-7] the CellSearch System is the only test approved by the Food and Drug Administration. The system has a sensitivity of approximately 65% for detecting circulating tumor cells (≥1 cell per 7.5 ml of blood) in patients with metastatic breast cancer.[8,9] Elevated levels of circulating tumor cells (defined as ≥5 cells per 7.5 ml of blood) have been associated with a worse prognosis.[8,10]

Circulating DNA fragments carrying tumor-specific sequence alterations (circulating tumor DNA) are found in the cell-free fraction of blood, representing a variable and generally small fraction of the total circulating DNA.[11,12] Advances in sequencing technologies have enabled the rapid identification of somatic genomic alterations in individual tumors, and these can be used to design personalized assays for the monitoring of circulating tumor DNA. Studies have shown the feasibility of using circulating tumor DNA to monitor tumor dynamics in a limited number of patients with various solid cancers, but few cases of breast cancer have been analyzed.[13-20] Here, we provide a direct comparison between circulating tumor DNA and other circulating biomarkers (CA 15-3 and circulating tumor cells) and medical imaging, the current standard of care, for the noninvasive monitoring of metastatic breast cancer.

Methods

Patients and Sample Collection

We carried out a prospective, single-center study to compare the sensitivity of measuring circulating tumor DNA, CA 15-3, and circulating tumor cells for monitoring tumor burden in patients with metastatic breast cancer (see the Supplementary Appendix, available with the full text of this article at NEJM.org). The study was approved by the local institutional research ethics committee.

Eligible patients were women with metastatic breast cancer currently undergoing active treatment. A total of 52 women were recruited, and 30 had genomic alterations suitable for monitoring. All women provided written informed consent. Serial blood samples (30 ml each) were collected between April 2010 and April 2012 at intervals of 3 or more weeks. Computed tomography (CT) was performed and reviewed in a blinded fashion to document response to treatment according to the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1.[21] All reagents and equipment used in the study were purchased.

Identification of Somatic Genomic Alterations

Sequencing was performed on DNA from breast-cancer specimens and matched normal tissue specimens, with the use of one or both of two methods: tagged-amplicon deep sequencing[22] for *PIK3CA* (encoding the phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha protein) and *TP53* (encoding tumor protein p53) or paired-end whole-genome sequencing (see the Supplementary Appendix). Tagged-amplicon deep sequencing was done by means of the Fluidigm Access Array and sequencing on the Illumina GAIIx or HiSeq instruments. Paired-end sequencing was done with the use of the Illumina HiSeq2000 instrument. Candidate mutations and structural variants were validated and confirmed to be somatic with the use of Sanger sequencing.

Isolation and Quantification of Circulating Tumor DNA

Blood samples that were collected in EDTA tubes were processed within 1 hour after collection and were centrifuged to separate the plasma from the peripheral-blood cells. DNA was extracted from

aliquots (2 ml) of plasma with the use of the QIAamp circulating nucleic acid kit (Qiagen). To measure the DNA carrying specific somatic genomic alterations in plasma, we carried out a microfluidic digital polymerase-chain-reaction (PCR) assay[17,23-25] (using the Fluidigm BioMark system) or direct plasma sequencing by means of tagged-amplicon deep sequencing[22] (using the Fluidigm Access Array and sequencing on the Illumina HiSeq2500 instrument) (see the Supplementary Appendix).

ASSAY OF CA 15-3 AND CIRCULATING TUMOR CELLS

We measured levels of CA 15-3 in aliquots (50 μl) of plasma by means of the ADVIA Centaur immunoassay system (Siemens Healthcare). Blood samples were collected in CellSave Preservative Tubes (Veridex) and were processed within 96 hours for the enumeration of circulating tumor cells with the use of the CellSearch System (Veridex). The counting of circulating tumor cells was performed in a manner blinded to the results of CT and assessments of CA 15-3 or circulating tumor DNA.

STATISTICAL ANALYSIS

To estimate the sensitivity of each of the circulating biomarkers, we used a modified bootstrapping method.[26] We randomly sampled the complete data set to obtain a new data set containing only one time point for each patient. This random sampling was repeated 1000 times to obtain 1000 data sets, each containing independent observations. For each data set, we calculated the sensitivity of each biomarker. The median sensitivity for each biomarker and the median difference in sensitivity between two biomarkers — circulating tumor DNA versus either CA 15-3 or circulating tumor cells — was then calculated across the 1000 data sets. The percentile method was used to obtain 95% confidence intervals.

Survival analysis was performed by fitting a different Cox regression model for each of the three variables of interest: circulating tumor DNA, circulating tumor cells, and CA 15-3. Each model was constructed with the use of the counting process notation (start, end, event),[27] such that for each time period, the date of the visit was taken as the start, and the date before the next visit (or the date of last follow-up) was considered the end. The predictors were modeled as time-dependent covariates that use splines to account for nonlinear relationships. Estimated survival curves were produced for different values of the covariates at the first visit. Wald statistic P values were reported for each model, and relative hazard plots were computed for each covariate, showing the linear predictor relative to the mean value of the covariate (for details, see the Supplementary Appendix).

RESULTS

IDENTIFICATION OF SOMATIC GENOMIC ALTERATIONS

Clinical details, results of CT imaging, and serial whole-blood samples were collected prospectively from 52 women undergoing therapy for metastatic breast cancer (Fig. 1, and Table S1 in the Supplementary Appendix). DNA extracted from archival-tumor tissue samples was analyzed to identify somatic genomic alterations, with the use of two approaches. First, we used targeted deep sequencing to screen for point mutations in *PIK3CA* and *TP53*,[28] which we identified in 25 of the 52 patients (Table S2 in the Supplementary Appendix). Second, we used whole-genome paired-end sequencing of tumor-tissue specimens and matched normal-tissue specimens in 9 of the 52 patients. We identified somatic structural variants[29] in 8 patients (Table S3 in the Supplementary Appendix), including 5 in whom no mutations were previously identified in *PIK3CA* or *TP53*, bringing the total number of patients with identified genomic alterations to 30 of 52 women (Fig. 1, and Fig. S1 in the Supplementary Appendix). In 3 patients, both mutations and structural variants were identified, enabling us to compare and contrast the use of point mutations[13] and structural variants[14,15] for serial monitoring of circulating tumor DNA. For 1 patient, we used whole-genome paired-end sequencing to identify multiple somatic mutations, enabling us to monitor multiple mutations in parallel in circulating tumor DNA (Table S2 in the Supplementary Appendix).

QUANTIFICATION OF CIRCULATING TUMOR DNA IN PLASMA

In the 30 women with somatic mutations or structural variants, circulating tumor DNA was quantified in a total of 141 serial plasma samples

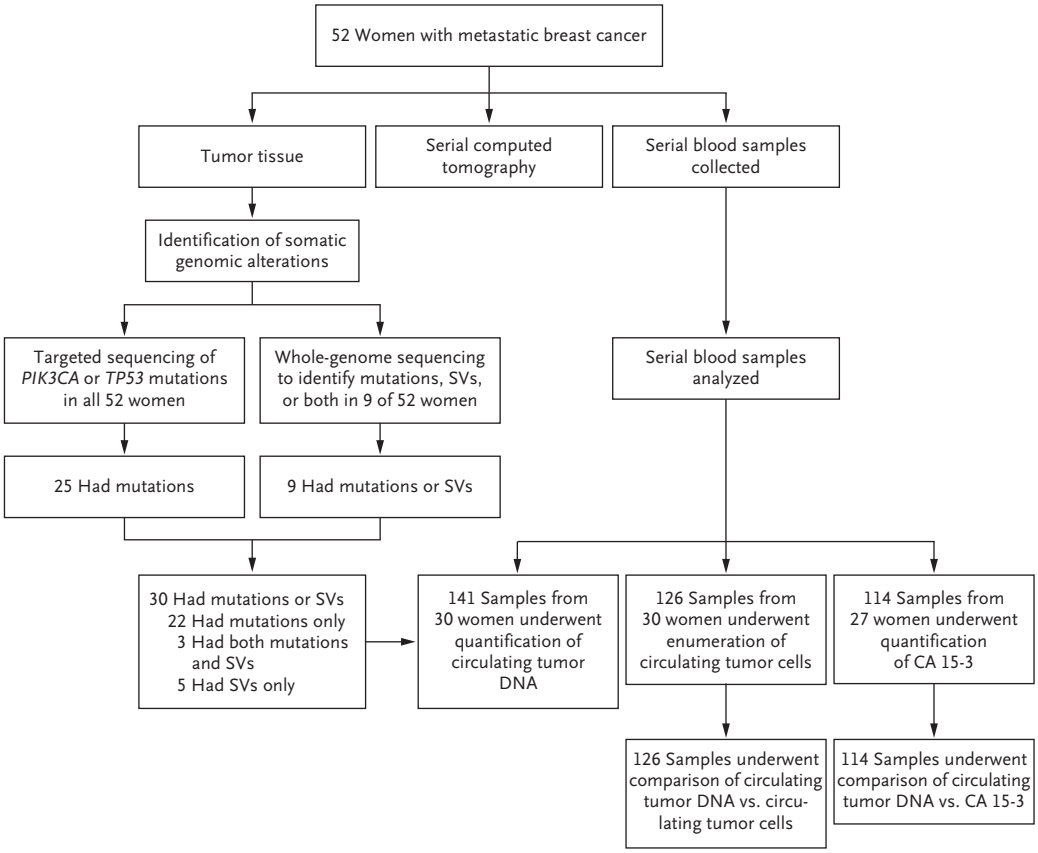


Figure 1. Enrollment of Patients and Collection of Clinical Samples.

In the 30 women who were found to have somatic mutations, structural variants (SVs), or both, the genomic alterations were determined through targeted deep sequencing or whole-genome paired-end sequencing of tumor-tissue specimens and matched normal-tissue specimens. CA 15-3 denotes cancer antigen 15-3.

by means of either digital PCR assay or tagged-amplicon deep sequencing.

Digital PCR assay was performed in 97 plasma samples from 19 of the 30 patients to track both somatic mutations and structural variants. The sensitivity of digital PCR assay allowed for the detection of a mutant allele fraction of 0.1% or more (one mutant molecule in a background of 1000 wild-type molecules) (Fig. S2 in the Supplementary Appendix).[17] Circulating tumor DNA was detected in 18 of the 19 women and in 80 of the 97 plasma samples (82%) analyzed.

As a high-throughput alternative to digital PCR assay, the remaining 44 plasma samples from the remaining 11 patients were analyzed with the use of tagged-amplicon deep sequencing.[22] The sensitivity of tagged-amplicon deep sequencing allowed for the detection of a mutant allele fraction of 0.14% or more with a confidence margin of 0.95.[22] Using this approach, circulating tumor DNA was identified in all 11 patients and in 35 of the 44 plasma samples (80%) analyzed.

In a subset of plasma samples in which circulating tumor DNA was analyzed by both techniques, quantification of mutant allele fraction by means of either tagged-amplicon deep sequencing or digital PCR assay showed excellent agreement (Fig. S3 in the Supplementary Appendix).[22] Taken together, circulating tumor DNA was detected in 29 of the 30 women (97%) and in 115 of the 141 plasma samples (82%). The median quantity of circulating tumor DNA across all samples was 150 amplifiable copies per milliliter of plasma (interquartile range, 9 to 720) (Table S4 in the Supplementary Appendix). The median mutant allele fraction was 4% (interquar-

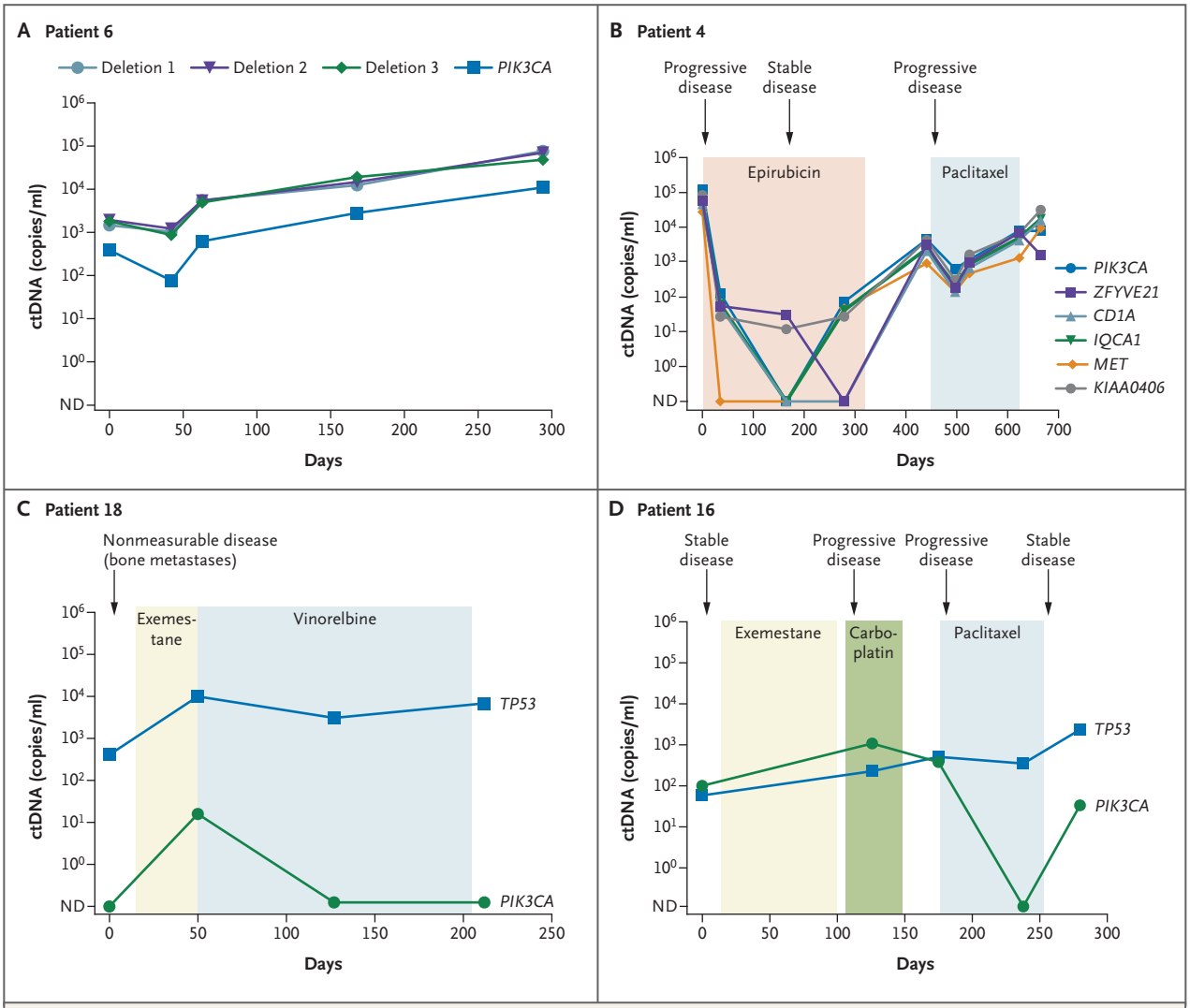


Figure 2. Monitoring Multiple Point Mutations and Structural Variants in Circulating DNA.

Panels A, B, and C show plasma levels of circulating tumor DNA (ctDNA) for three patients (one per panel), quantified in parallel by means of a digital polymerase-chain-reaction (PCR) assay across multiple time points. In Panels B, C, and D, the use of endocrine or cytotoxic therapy is indicated by colored shading, and disease status at various times (as ascertained on computed tomography) is shown. Panel A shows three structural variants (deletions) and a point mutation in *PIK3CA*. The three deletions occurred in the setting of a complex rearrangement associated with amplification. Panel B shows six point mutations, all of which showed similar dynamic patterns. Panel C shows point mutations in *PIK3CA* and *TP53*; the *TP53* mutation was dominant in the circulation as compared with the *PIK3CA* mutation. Panel D shows plasma levels of ctDNA for a fourth patient, with point mutations in *PIK3CA* and *TP53* quantified by means of tagged-amplicon deep sequencing. The *TP53* mutation was identified in plasma only, and levels remained elevated after paclitaxel chemotherapy despite a fall in the *PIK3CA* mutation level in the presence of stable disease. ND denotes not detected.

tile range, 1 to 14). The 1 patient in whom circulating tumor DNA was not detected (Patient 12) had a low burden of metastatic disease (small-volume mediastinal lymphadenopathy) and no evidence of disease progression during the study. Overall, levels of total plasma DNA were measured in parallel and had limited informative content (Fig. S4 in the Supplementary Appendix).

CONCURRENT MONITORING OF MULTIPLE SOMATIC GENOMIC ALTERATIONS IN PLASMA

Plasma levels of either mutations or structural variants identified in the tumor tissue of the same patient (Fig. S1C in the Supplementary Appendix) showed a similar dynamic pattern (Fig. 2A, and Table S4 in the Supplementary Appendix). This confirmed the utility and comparability of both

approaches. In women with tumors in which the genomic location of the structural variants overlapped with an amplified locus, such alterations were detected in the plasma at higher concentrations, confirming that the assay of circulating tumor DNA is quantitative (Fig. 2A, and Fig. S1B and Table S5 in the Supplementary Appendix).

When multiple mutations were identified in tumor-tissue samples (Fig. S1C in the Supplementary Appendix), they generally showed similar dynamic patterns in plasma (Fig. 2B, and Table S4 in the Supplementary Appendix). However, in some cases, we also observed evidence of clonal heterogeneity, whereby certain mutations dominated in the plasma (Fig. 2C, and Table S4 in the Supplementary Appendix). Tagged-amplicon deep sequencing also identified mutations in plasma that were not detected in archival-tumor DNA (Fig. S1C in the Supplementary Appendix).[22] In these cases, the archival primary tissue had been collected more than 10 years previously, and the discordance may have reflected tumor evolution.[30,31] These mutations showed diverging patterns over the course of disease progression and treatment (Fig. 2D, and Table S4 in the Supplementary Appendix), as compared with the mutations identified in the tumor, suggesting that they originated from different subclones.

SENSITIVITY OF CIRCULATING TUMOR DNA, CA 15-3, AND CIRCULATING TUMOR CELLS

Data comparing CA 15-3 values and circulating tumor DNA levels were available across 114 serial time points for 27 patients (Fig. 3A, and Table S4 in the Supplementary Appendix). CA 15-3 levels were elevated (>32.4 U per milliliter) at one or more time points in 21 of the 27 women (78%) and in 71 of the 114 samples (62%). In contrast, circulating tumor DNA was detected in 26 of 27 women (96%) and in 94 of 114 samples (82%). Of the 43 samples without elevated CA 15-3 levels, 27 (63%) had measurable levels of circulating tumor DNA. Using a modified bootstrapping method, we showed improved sensitivity of circulating tumor DNA as compared with CA 15-3 (85% vs. 59%), with a median difference in sensitivity of 26% (95% confidence interval [CI], 11 to 37; P<0.002).

Circulating tumor cells were quantified by means of the CellSearch System at 126 time points for all 30 women (Fig. 3B, and Table S4 in the Supplementary Appendix). Circulating tumor cells (≥1 cell per 7.5 ml of blood) were detected at one or more time points in 26 of the 30 women (87%), and elevated circulating tumor cells (≥5 cells per 7.5 ml of blood) were identified in 18 of the 30 women (60%). Of the 126 samples, 50 (40%) had no detected circulating tumor cells, and 76 (60%) had 1 or more cells per 7.5 ml, of which 46 (37% of all 126 samples) had 5 or more cells per 7.5 ml. In contrast, circulating tumor DNA was detected in 29 of the 30 women (97%) and at 106 of 126 time points (84%). In the 50 samples in which no circulating tumor cells were detected, 33 (66%) had measurable levels of circulating tumor DNA. According to the modified bootstrapping method, circulating tumor DNA had sensitivity superior to that of circulating tumor cells (90% vs. 67%), with a median difference in sensitivity of 27% (95% CI, 13 to 37; P<0.002). At the median, the number of amplifiable copies of circulating tumor DNA was 133 times the number of circulating tumor cells and had a greater dynamic range (Fig. 3B).

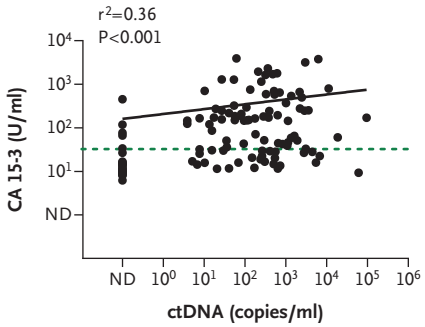
CT AND CIRCULATING BIOMARKERS FOR TUMOR MONITORING

We compared the performance of circulating biomarkers with the performance of CT in 20 patients with measurable disease (as defined by RECIST[21]) and for whom circulating biomarker data were available at 3 or more time points over a period of more than 100 days of follow-up (Fig. S5 in the Supplementary Appendix). Circulating tumor DNA was detected and showed serial changes in 19 of 20 women (95%) with fluctuations in circulating tumor DNA generally correlating with treatment responses seen on imaging (Fig. 4A, and Fig. S5 in the Supplementary Appendix). Similar findings were noted for women with 5 or more circulating tumor cells per 7.5 ml of blood (10 of 20 patients [50%]) in which serial changes in circulating tumor cell counts were evident and corresponded with responses ascertained on CT (Fig. 4A). However, in the remaining 10 women with a maximal count of circulating tumor cells of fewer than 5 cells per 7.5 ml of blood, the number of circulating tumor cells was uninformative (Fig. 4B and 4C, and Fig. S5 in the Supplementary Appendix).

Similar to the findings regarding circulating tumor cells was the finding that women with high levels of CA 15-3 had fluctuations corresponding to responses on imaging but with a smaller dynamic range (Fig. 4A and 4B, and Fig.

A CA 15-3 vs. ctDNA

CA 15-3	ctDNA		
	Detected	Not detected	Total
Patients			
Elevated (>32.4 U/ml)	21	0	21
Not elevated (≤32.4 U/ml)	5	1	6
Total	26	1	27
ctDNA sensitivity, 26/27 (96%) CA 15-3 sensitivity, 21/27 (78%)			
Samples			
Elevated (>32.4 U/ml)	67	4	71
Not elevated (≤32.4 U/ml)	27	16	43
Total	94	20	114
ctDNA sensitivity, 94/114 (82%) CA 15-3 sensitivity, 71/114 (62%)			



B CTC vs. ctDNA

CTC	ctDNA		
	Detected	Not detected	Total
Patients			
Elevated (≥5)	18	0	18
Detected (1–4)	7	1	8
Not detected (0)	4	0	4
Total	29	1	30
ctDNA sensitivity, 29/30 (97%) CTC sensitivity (detected, >0), 26/30 (87%) CTC sensitivity (elevated, ≥5), 18/30 (60%)			
Samples			
Elevated (≥5)	45	1	46
Detected (1–4)	28	2	30
Not detected (0)	33	17	50
Total	106	20	126
ctDNA sensitivity, 106/126 (84%) CTC sensitivity (detected, >0), 76/126 (60%) CTC sensitivity (elevated, ≥5), 46/126 (37%)			
Median ratio of ctDNA copy numbers (per 3.75 ml of plasma) to number of CTCs (per 7.5 ml of whole blood)=133 (interquartile range, 51–528)			

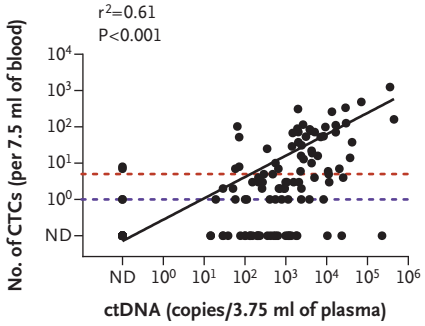
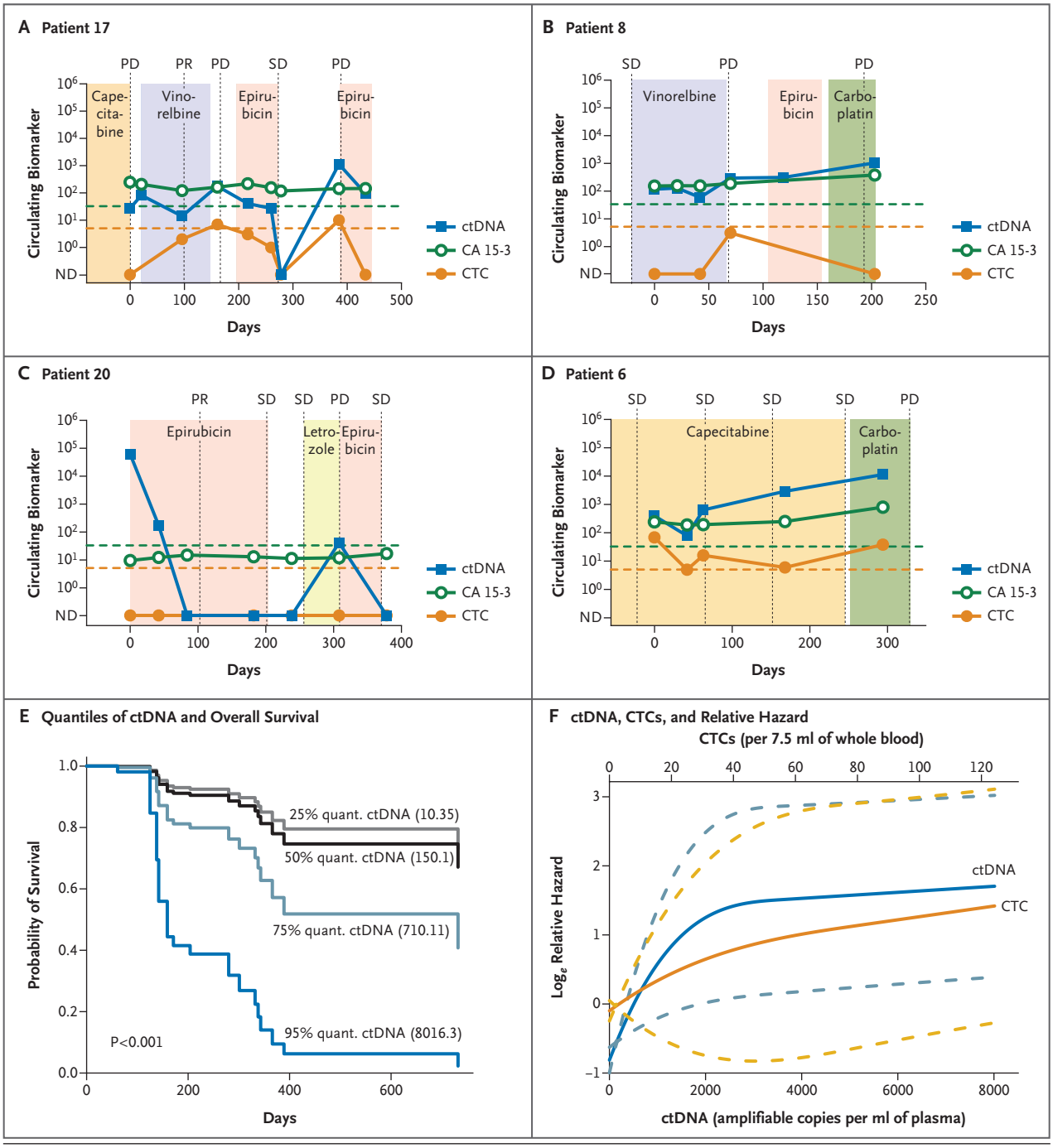


Figure 3. Comparison of Circulating Tumor DNA, CA 15-3, and Circulating Tumor Cells as Blood-Based Biomarkers.

Panel A shows comparisons of CA 15-3 levels (U per milliliter of plasma) and circulating tumor DNA (ctDNA) levels (amplifiable copies per milliliter of plasma) across the maximal value analyzed for individual patients and across all samples analyzed for all patients. The green horizontal dashed line indicates the CA 15-3 threshold of 32.4 U per milliliter. The Spearman correlation coefficient (r) between CA 15-3 levels and ctDNA levels across all time points was 0.36 (P<0.001). Panel B shows comparisons of circulating tumor cell (CTC) numbers (per 7.5 ml of whole blood) and ctDNA numbers (amplifiable copies per 3.75 ml of plasma) across the maximal value analyzed for individual patients and across all samples analyzed for all patients. Copy numbers of ctDNA were adjusted for direct comparison to the numbers of circulating tumor cells from an equivalent volume of whole blood (7.5 ml). The purple dashed line indicates the CTC threshold of 1 cell per 7.5 ml of blood, and the orange dashed line indicates the CTC threshold of 5 cells per 7.5 ml of blood. The Spearman correlation coefficient (r) between quantified ctDNA levels and numbers of CTCs across all time points was 0.61 (P<0.001). ND denotes not detected.

S5 in the Supplementary Appendix). In patients with levels of CA 15-3 of 50 U or less per milliliter (8 of 19 patients [42%]), no consistent serial changes in CA 15-3 levels were seen (Fig. 4C, and Fig. S5 in the Supplementary Appendix).

Progressive disease was documented on CT (as defined by RECIST) in 19 of 20 women during the follow-up period; CA 15-3 data were available for 18 of these women (95%) (Fig. S5 in the Supplementary Appendix). Increases in circulating tumor DNA levels reflected progressive disease in 17 of the 19 women (89%). In these women, on average, circulating tumor DNA levels increased by a factor of 505 (range, 2 to 4457) from the nadir before the establishment of progressive disease. The numbers of circulating tu-



mor cells increased in 7 of the 19 women (37%), and CA 15-3 levels increased in 9 of 18 women (50%) (Fig. S5 in the Supplementary Appendix). In 10 of the 19 patients (53%), levels of circulating tumor DNA increased at one or more consecutive time points, on average 5 months (range, 2 to 9) before the establishment of progressive disease by means of imaging (Fig. 4D, and Fig. S5 in the Supplementary Appendix). In 2 women (Patients 9 and 22), increasing levels of circulating tumor DNA did not reflect the presence of progressive disease as assessed on CT (a detailed description

Figure 4 (facing page). Comparison of Circulating Biomarkers to Monitor Tumor Dynamics and Predict Survival.

Panels A, B, C, and D show serial circulating tumor DNA (ctDNA) levels (number of copies per milliliter of plasma), circulating tumor cell (CTC) numbers (per 7.5 ml of whole blood), CA 15-3 levels (U per milliliter), and disease status as ascertained on computed tomography (vertical dashed lines) for four patients (one in each panel). Details of endocrine or cytotoxic therapy are indicated by colored shading. The orange dashed line indicates the threshold of 5 CTCs per 7.5 ml of whole blood. The green dashed line indicates the CA 15-3 threshold of 32.4 U per milliliter. ND denotes not detected, PD progressive disease, PR partial response, and SD stable disease. Panel E shows the results of a Cox regression model, which identified an inverse relationship between quantiles (quant.) of ctDNA (indicated in copies per milliliter of plasma) and overall survival, with increasing levels significantly associated with poor overall survival ($P<0.001$). At 200, 400, and 600 days, a total of 23, 8, and 3 patients were at risk, respectively. Panel F shows that increasing ctDNA levels (copies per milliliter), as indicated on the bottom x axis, and increasing numbers of CTCs (per 7.5 ml of whole blood), as indicated on the top x axis, were associated with an increased $\log_e$ relative hazard. The prognostic discrimination power of circulating tumor DNA level was greatest with levels up to 2000 copies per milliliter. Patients with levels of more than 2000 copies per milliliter were uniformly found to have the worst prognosis. The prognostic power of CTCs increased according to the number of cells. Dashed lines represent 95% confidence intervals.

of these patients is provided in Fig. S5 in the Supplementary Appendix).

PROGNOSTIC USE OF CIRCULATING BIOMARKERS

Finally, we compared the circulating biomarkers with respect to prognostic use. Using a Cox proportional-hazards model in which circulating tumor DNA was treated as a continuous time-dependent variable, we found that increasing levels of circulating tumor DNA were associated with inferior overall survival ($P<0.001$) (Fig. 4E). Circulating tumor cells were also found to have prognostic significance ($P=0.03$) (Fig. S6A in the Supplementary Appendix). In contrast, CA 15-3 was not found to be prognostic in this series of patients (Fig. S6B in the Supplementary Appendix). Increasing numbers of circulating tumor cells and increasing levels of circulating tumor DNA were associated with an increased hazard (Fig. 4F), indicating that absolute levels of each is informative in guiding prognosis.

DISCUSSION

In the detection of metastatic breast cancer, circulating tumor DNA shows superior sensitivity to that of other circulating biomarkers and has a greater dynamic range that correlates with changes in tumor burden. Circulating tumor DNA often provides the earliest measure of treatment response, as has been supported by recent analyses of circulating tumor DNA in other solid cancers.[20,32]

The monitoring of circulating tumor DNA levels requires the identification of somatic alterations in individual patients. Future developments will reduce the cost of whole-genome paired-end sequencing, and targeted sequencing can be readily expanded to include other genes, in addition to *PIK3CA* and *TP53*, known to be recurrently mutated in breast cancer.[33-35] Here we have demonstrated the use of two strategies to quantify circulating tumor DNA: digital PCR assay and targeted deep sequencing. Digital PCR assay provides high accuracy and sensitivity but requires the design of personalized assays, an expensive and rate-limiting step. Targeted deep sequencing of plasma DNA provides a cost-effective alternative for high-throughput analysis and may overcome limitations of initial tumor-tissue assessment by virtue of allowing for the direct identification of mutations in plasma.[22] However, our findings on circulating tumor DNA are not limited to these molecular platforms. Other methods for the identification of somatic mutations (such as exome sequencing[33]) or for the quantification of circulating tumor DNA (e.g., BEAMing [beads, emulsions, amplification, and magnetics] technology[13] or Safe-SeqS [Safe-Sequencing System][36]) may be applied with even greater sensitivity. Recent studies have also shown the feasibility of performing genomewide analysis of tumor-associated copy-number changes and mutations in plasma.[37-39]

Our expanding knowledge of the genetic mechanisms underpinning breast cancer now provides a framework to better stratify patients.[30,33-35,40,41] The analysis of circulating tumor DNA represents a unique opportunity to integrate this knowledge into the clinical arena. Although the acquisition of tumor-tissue specimens will continue to be important, the use of biopsy specimens is limited, since such material may

not capture tumor heterogeneity; in addition, repeated biopsy is impractical. Circulating tumor DNA represents a "liquid biopsy" alternative, allowing for sensitive and specific serial sampling to be performed during the course of treatment.

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REFERENCES

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010;127:2893-917.
2. Lauro S, Trasatti L, Bordin F, et al. Comparison of CEA, MCA, CA 15-3 and CA 27-29 in follow-up and monitoring therapeutic response in breast cancer patients. Anticancer Res 1999;19:3511-5.
3. Harris L, Fritsche H, Mennel R, et al. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. J Clin Oncol 2007;25:5287-312.
4. Duffy MJ, Evoy D, McDermott EW. CA 15-3: uses and limitation as a biomarker for breast cancer. Clin Chim Acta 2010;411:1869-74.
5. Lianidou ES, Markou A. Circulating tumor cells in breast cancer: detection systems, molecular characterization, and future challenges. Clin Chem 2011;57:1242-55.
6. Nagrath S, Sequist LV, Maheswaran S, et al. Isolation of rare circulating tumour cells in cancer patients by microchip technology. Nature 2007;450:1235-9.
7. Pantel K, Brakenhoff RH, Brandt B. Detection, clinical relevance and specific biological properties of disseminating tumour cells. Nat Rev Cancer 2008;8:329-40.
8. Cristofanilli M, Budd GT, Ellis MJ, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med 2004;351:781-91.
9. Mego M, De Giorgi U, Dawood S, et al. Characterization of metastatic breast cancer patients with nondetectable circulating tumor cells. Int J Cancer 2011;129:417-23.
10. Pierga JY, Hajage D, Bachelot T, et al. High independent prognostic and predictive value of circulating tumor cells compared with serum tumor markers in a large prospective trial in first-line chemotherapy for metastatic breast cancer patients. Ann Oncol 2012;23:618-24.
11. Schwarzenbach H, Hoon DS, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer 2011;11:426-37.
12. Gormally E, Caboux E, Vineis P, Hainaut P. Circulating free DNA in plasma or serum as biomarker of carcinogenesis: practical aspects and biological significance. Mutat Res 2007;635:105-17.
13. Diehl F, Schmidt K, Choti MA, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med 2008;14:985-90.
14. Leary RJ, Kinde I, Diehl F, et al. Development of personalized tumor biomarkers using massively parallel sequencing. Sci Transl Med 2010;2:20ra14.
15. McBride DJ, Orpana AK, Sotiriou C, et al. Use of cancer-specific genomic rearrangements to quantify disease burden in plasma from patients with solid tumors. Genes Chromosomes Cancer 2010;49:1062-9.
16. Chen X, Bonnefoi H, Diebold-Berger S, et al. Detecting tumor-related alterations in plasma or serum DNA of patients diagnosed with breast cancer. Clin Cancer Res 1999;5:2297-303.
17. Yung TK, Chan KC, Mok TS, Tong J, To KF, Lo YM. Single-molecule detection of epidermal growth factor receptor mutations in plasma by microfluidics digital PCR in non-small cell lung cancer patients. Clin Cancer Res 2009;15:2076-84.
18. Nakamura T, Sueoka-Aragane N, Iwanaga K, et al. A noninvasive system for monitoring resistance to epidermal growth factor receptor tyrosine kinase inhibitors with plasma DNA. J Thorac Oncol 2011;6:1639-48.
19. Otsuka J, Okuda T, Sekizawa A, et al. Detection of p53 mutations in the plasma DNA of patients with ovarian cancer. Int J Gynecol Cancer 2004;14:459-64.
20. Diaz LA Jr, Williams RT, Wu J, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature 2012;486:537-40.
21. Eisenhauer EA, Therasse P, Bogaerts J, et al. New Response Evaluation Criteria in Solid Tumours: revised RECIST guideline (version 1.1). Eur J Cancer 2009;45:228-47.
22. Forshew T, Murtaza M, Parkinson C, et al. Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. Sci Transl Med 2012;4:136ra68.
23. Vogelstein B, Kinzler KW. Digital PCR. Proc Natl Acad Sci U S A 1999;96:9236-41.
24. Wang J, Ramakrishnan R, Tang Z, et al. Quantifying EGFR alterations in the lung cancer genome with nanofluidic digital PCR arrays. Clin Chem 2010;56:623-32.
25. Lo YM, Lun FM, Chan KC, et al. Digital PCR for the molecular detection of fetal chromosomal aneuploidy. Proc Natl Acad Sci U S A 2007;104:13116-21.
26. Efron B, Tibshirani R. An introduction to the bootstrap. London: Chapman & Hall/CRC, 1993.
27. Andersen PK, Gill RD. Cox's regression model for counting processes: a large sample study. Ann Stat 1982;10:1100-20.
28. Forbes SA, Bindal N, Bamford S, et al. COSMIC: mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer. Nucleic Acids Res 2011;39:D945-D950.
29. Stephens PJ, McBride DJ, Lin ML, et al. Complex landscapes of somatic rearrangement in human breast cancer genomes. Nature 2009;462:1005-10.
30. Shah SP, Roth A, Goya R, et al. The clonal and mutational evolution spectrum of primary triple-negative breast cancers. Nature 2012;486:395-9.
31. Higgins MJ, Jelovac D, Barnathan E, et al. Detection of tumor PIK3CA status in metastatic breast cancer using peripheral blood. Clin Cancer Res 2012;18:3462-9.
32. Misale S, Yaeger R, Hobor S, et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature 2012;486:532-6.
33. Stephens PJ, Tarpey PS, Davies H, et al. The landscape of cancer genes and mutational processes in breast cancer. Nature 2012;486:400-4.
34. Banerji S, Cibulskis K, Rangel-Escareno C, et al. Sequence analysis of mutations and translocations across breast cancer subtypes. Nature 2012;486:405-9.
35. The Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. Nature 2012;490:61-70.
36. Kinde I, Wu J, Papadopoulos N, Kinzler KW, Vogelstein B. Detection and quantification of rare mutations with

massively parallel sequencing. Proc Natl Acad Sci U S A 2011;108:9530-5.
37. Chan KC, Jiang P, Zheng YW, et al. Cancer genome scanning in plasma: detection of tumor-associated copy number aberrations, single-nucleotide variants, and tumoral heterogeneity by massively parallel sequencing. Clin Chem 2013;59:211-24.
38. Shaw JA, Page K, Blighe K, et al. Genomic analysis of circulating cell-free DNA infers breast cancer dormancy. Genome Res 2012;22:220-31.
39. Leary RJ, Sausen M, Kinde I, et al. Detection of chromosomal alterations in the circulation of cancer patients with whole-genome sequencing. Sci Transl Med 2012;4:162ra154.
40. Ellis MJ, Ding L, Shen D, et al. Whole-genome analysis informs breast cancer response to aromatase inhibition. Nature 2012;486:353-60.
41. Curtis C, Shah SP, Chin SF, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature 2012;486:346-52.