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

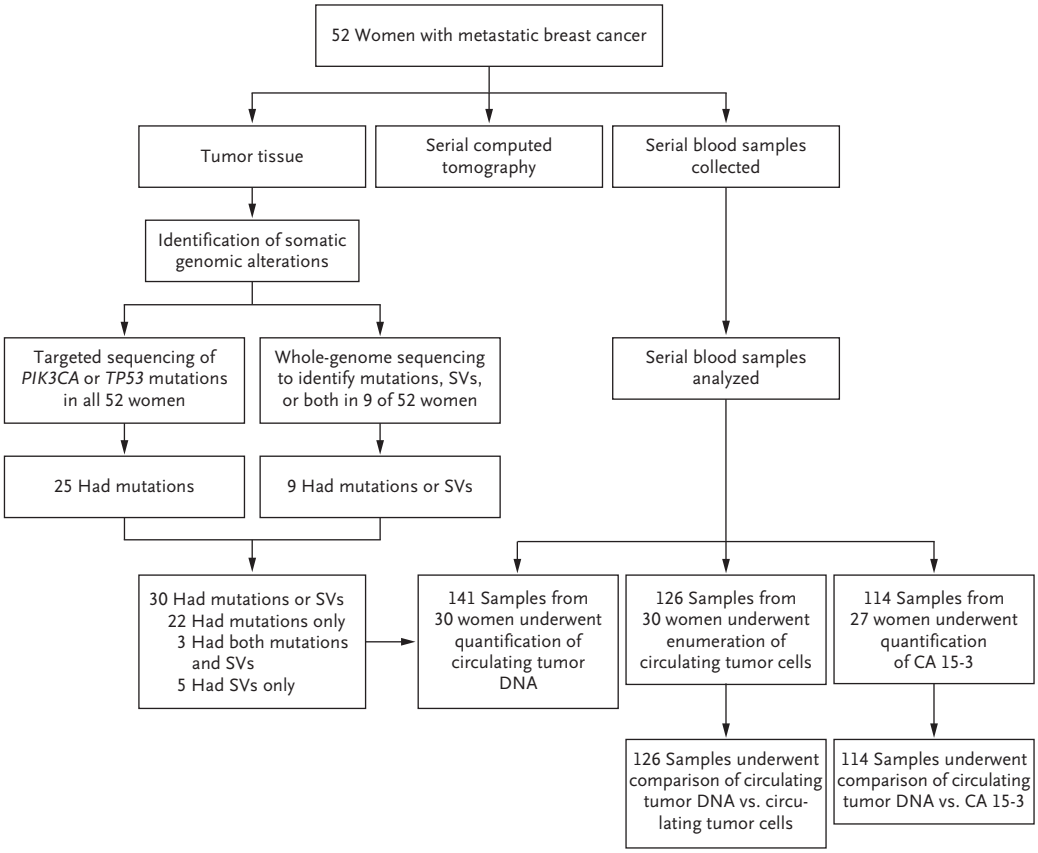

Figure 1. Enrollment of Patients and Collection of Clinical Samples.

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-

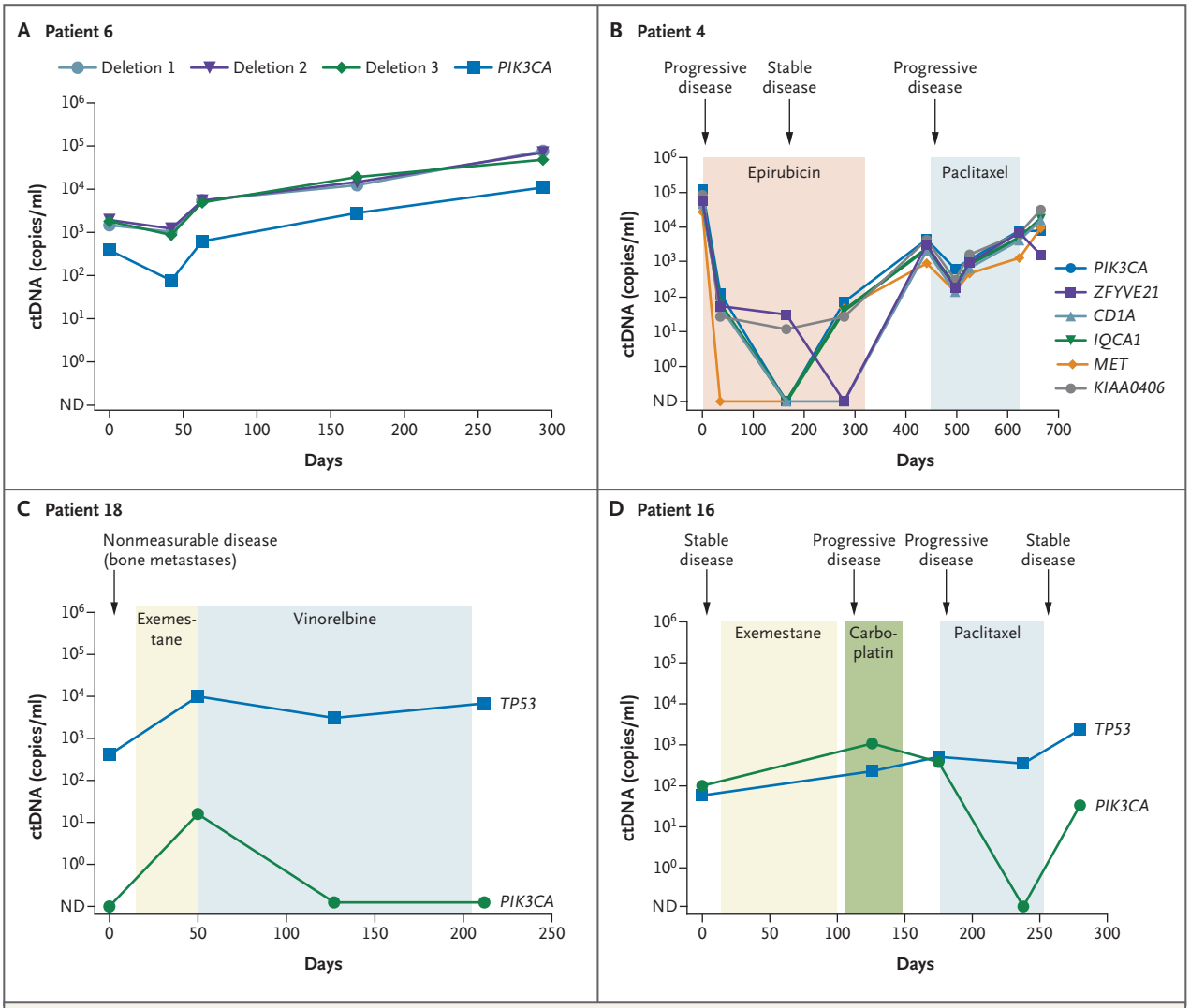

Figure 2. Monitoring Multiple Point Mutations and Structural Variants in Circulating DNA.

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

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