CANCER

Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer

Isaac Garcia-Murillas,[1]* Gaia Schiavon,[1,2]*† Britta Weigelt,[3] Charlotte Ng,[3] Sarah Hrebien,[1] Rosalind J. Cutts,[1] Maggie Cheang,[4] Peter Osin,[2] Ashutosh Nerurkar,[2] Iwanka Kozarewa,[1] Javier Armisen Garrido,[1] Mitch Dowsett,[1,2] Jorge S. Reis-Filho,[3] Ian E. Smith,[2] Nicholas C. Turner[1,2]‡

The identification of early-stage breast cancer patients at high risk of relapse would allow tailoring of adjuvant therapy approaches. We assessed whether analysis of circulating tumor DNA (ctDNA) in plasma can be used to monitor for minimal residual disease (MRD) in breast cancer. In a prospective cohort of 55 early breast cancer patients receiving neoadjuvant chemotherapy, detection of ctDNA in plasma after completion of apparently curative treatment—either at a single postsurgical time point or with serial follow-up plasma samples—predicted metastatic relapse with high accuracy [hazard ratio, 25.1 (confidence interval, 4.08 to 130.5; log-rank $P < 0.0001$) or 12.0 (confidence interval, 3.36 to 43.07; log-rank $P < 0.0001$), respectively]. Mutation tracking in serial samples increased sensitivity for the prediction of relapse, with a median lead time of 7.9 months over clinical relapse. We further demonstrated that targeted capture sequencing analysis of ctDNA could define the genetic events of MRD, and that MRD sequencing predicted the genetic events of the subsequent metastatic relapse more accurately than sequencing of the primary cancer. Mutation tracking can therefore identify early breast cancer patients at high risk of relapse. Subsequent adjuvant therapeutic interventions could be tailored to the genetic events present in the MRD, a therapeutic approach that could in part combat the challenge posed by intratumor genetic heterogeneity.

INTRODUCTION

Breast cancer is the most frequently diagnosed cancer worldwide and, in women, the second most common cause of cancer deaths (*1*). About 95% of women with breast cancer present with early-stage cancer without macroscopic evidence of metastases (*2*). In many women, however, breast cancer has already metastasized at diagnosis, and such micrometastatic disease can, in time, result in overt metastatic recurrence. The identification of those patients who have residual micrometastatic disease or minimal residual disease (MRD) that has not been eradicated by adjuvant systemic therapy and surgery would allow for the development of clinical trials of adjuvant therapies to prevent relapse focused on those who are at highest risk.

Circulating tumor DNA (ctDNA) can be detected in the plasma and serum of patients with advanced cancer (*3*), acting as a potential noninvasive source to characterize the somatic genetic features of their tumors (*4*–*7*). Limited data are available on whether ctDNA analyses would be applicable to early cancer (*8*, *9*), in part because the low tumor burden of micrometastatic disease makes detection of ctDNA challenging, as ctDNA is often detectable at a very low level in plasma DNA (*10*, *11*).

Here, we assess the potential to detect ctDNA in early-stage, primary breast cancer. We demonstrate that ctDNA detection with personalized digital polymerase chain reaction (dPCR) assays of somatic mutations can be used to identify MRD and that such MRD detection with plasma ctDNA can accurately identify patients at risk of cancer relapse. After detection of patient ctDNA with dPCR mutation tracking, we further asked whether high-depth targeted massively parallel sequencing (MPS) of ctDNA could be used to interrogate the genetics of MRD as a potential route to identify the lethal clone in genetically heterogeneous cancers.

RESULTS

Personalized tumor-specific dPCR assays for mutation tracking

We investigated the potential utility of ctDNA analysis in early breast cancer in a prospectively accrued cohort of 55 women presenting with early-stage breast cancer who received neoadjuvant chemotherapy before surgery (Fig. 1). We subjected primary tumor DNA, extracted from a tumor biopsy at diagnosis before treatment, to MPS, identifying one or more somatic mutation(s) in 78% [43 of 55; 95% confidence interval (CI), 65 to 88%] of cancers, with two or more mutations found in 12 cases.

To track these mutations in plasma DNA and identify the presence of ctDNA, we designed personalized dPCR assays for each somatic mutation identified (table S1). dPCR can accurately quantify mutant DNA at single-molecule sensitivity, even in the presence of vast amounts of wild-type (WT) DNA (Fig. 1) (*12*, *13*). MPS and dPCR analysis had a high level of agreement in baseline tumor DNA in the assessment of the mutant allele fractions (Fig. 2A), demonstrating the robust ability to develop dPCR assays for diverse mutations. For a representative number of cases ($n = 9$), the estimation of mutation frequency in plasma DNA was highly correlated in replicate dPCR assays ($r^2 = 0.98$) (Fig. 2B). In patients with two mutations identified in the primary tumor, we tracked both mutations in plasma with 96% agreement for present/absent

[1]Breakthrough Breast Cancer Research Centre, The Institute of Cancer Research, Fulham Road, London SW3 6JB, UK. [2]Breast Unit, Royal Marsden Hospital, Fulham Road, London SW3 6JJ, UK. [3]Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA. [4]Institute of Cancer Research Clinical Trials and Statistics Unit, The Institute of Cancer Research, 15 Cotswold Road, Surrey SM2 5NG, UK.
*These authors contributed equally to this work.
†Present address: Translational Science, Oncology iMed, AstraZeneca, Cambridge CB4 0WG, UK.
‡Corresponding author. E-mail: nicholas.turner@icr.ac.uk

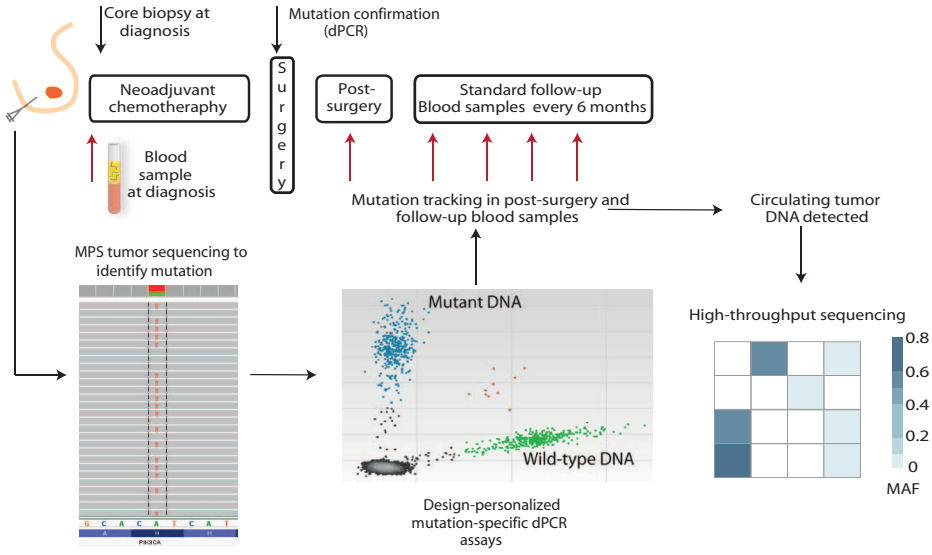

Fig. 1. Personalized dPCR assays for mutation tracking of ctDNA in plasma of patients with early breast cancer. The baseline biopsy of patients presenting with early breast cancer was subjected to targeted MPS to identify somatic (tumor-specific) mutations. Personalized, patient-specific dPCR assays were developed to detect the mutation in plasma DNA that was extracted from samples taken at baseline, after surgery, and serially every 6 months during follow-up (mutation tracking). After detection of ctDNA with dPCR mutation tracking, plasma samples were subjected to high-depth targeted capture MPS to interrogate the repertoire of somatic genetic alterations in detected MRD. MAF, mutant allele frequency.

mutation calling in the same plasma DNA sample (κ = 0.92; 95% CI, 0.77 to 1.0), emphasizing the reproducible and robust nature of the assays developed.

Tracking mutations in ctDNA to identify MRD and anticipate relapse

We next assessed whether dPCR could be used for the detection of ctDNA to predict early relapse. The personalized dPCR assays were used to track mutations in serial plasma samples taken at baseline, postoperatively with the sample taken 2 to 4 weeks after surgery, and then every 6 months during follow-up (Fig. 2C and fig. S1). Two patients whose tumors harbored the same *PIK3CA* c.3140A>T (p.H1047L) somatic mutation illustrated the potential of mutation tracking. In patient A310001, who remained disease-free at 30 months postsurgical follow-up, the mutation was undetected in all follow-up plasma samples, suggesting clearance of tumor by neoadjuvant chemotherapy and surgery. In contrast, in patient A310006, ctDNA was detected in the postsurgical sample, identifying the presence of MRD. At 6.2 months after surgery, there was a marked increase in mutation abundance, suggesting increasing disease burden, followed by clinical relapse 8.1 months after surgery with widespread metastatic disease.

We assessed the potential to predict relapse from the different time points of ctDNA analysis, starting with the baseline plasma sample taken at diagnosis before any treatment. Consistent with previous observations (*9*), ctDNA was detected in 69% (29 of 42; 95% CI, 53% to 82%) of baseline plasma samples (Fig. 3). The level of baseline ctDNA was associated with markers of disease aggressiveness, such as histological grade and estrogen receptor (ER)–negative status, but not primary tumor size (table S2). ctDNA detection at baseline, before any treatment, was not predictive of disease-free survival (Fig. 3A), and ctDNA abundance at baseline was not statistically associated with early relapse (median of 13.8 versus 3.13 mutations/ml for early relapse and disease-free survival, respectively) (Fig. 3B).

We next assessed the potential of a single postsurgical sample taken 2 to 4 weeks after surgery. ctDNA was detected in the single postoperative blood test in 19% (7 of 37; 95% CI, 8 to 35%) of patients (Fig. 4A), with highly variable mutational load (median of 19.2 copies/ml; range, 1.8 to 6284 copies/ml). In these samples, ctDNA detection was predictive of early relapse [disease-free survival: median of 6.5 months (ctDNA detected) versus median not reached (ctDNA not detected); hazard ratio (HR), 25.1 (95% CI, 4.08 to 130.5)] (Fig. 4A), with a concordance index (C-index) of 0.78. Detection of ctDNA in a single postsurgical sample was a significant predictor of early relapse in a multivariable model (table S3). Six patients did not have a sample taken at 2 to 4 weeks after surgery and were excluded from analysis of this time point.

We examined whether the detection of ctDNA in serial samples, which we termed "mutation tracking," could improve relapse prediction compared with a single postsurgical sample (Fig. 4B). Detection of ctDNA in serial samples was predictive of early relapse [disease-free survival: median of 13.6 months (ctDNA detected) versus median not reached (ctDNA not detected); HR, 12.0 (95% CI, 3.36 to 43.07)], with a C-index of 0.75. Detection of ctDNA by mutation tracking was a significant predictor of early relapse in a multivariable model (table S4).

Sensitivity in a single postsurgical sample was limited by patients who had undetectable ctDNA in the single postsurgical plasma sample and required serial sampling to detect ctDNA. Of the patients who relapsed in the follow-up period, 50% (6 of 12) had ctDNA detected in the single postsurgical sample and 80% (12 of 15) had ctDNA detected by mutation tracking. Of the patients who did not relapse, 96% did not have ctDNA detected in either the single postsurgical sample (24 of 25) or by mutation tracking (27 of 28) ($P = 0.0038$ for single postsurgical samples; $P < 0.0001$ for mutation tracking in serial samples, χ^2 test with Yates' correction). One patient with ctDNA detectable after surgery did not relapse in the follow-up period (A310033) (Fig. 5A). This patient with triple-negative breast cancer had ctDNA detectable in the first postsurgical sample, with a subsequent increase in ctDNA abundance in serial sampling.

Detection of ctDNA had a median of 7.9 months (range, 0.03 to 13.6 months) lead time over clinical relapse, identifying the presence of MRD that was not detectable on conventional imaging for patient A310004 (Fig. 5B). ctDNA did not disappear in serial sampling of plasma from patients with early relapse (Fig. 5C). Both assessment of ctDNA in the single postoperative sample and mutation tracking in serial samples appeared to predict relapse in all the major subtypes of breast cancer (Fig. 6), although mutation tracking with serial samples was more sensitive in ER^+ breast cancer.

In the three patients whose metastatic relapse was restricted to the brain, no ctDNA was detectable by dPCR before or at relapse. Patients

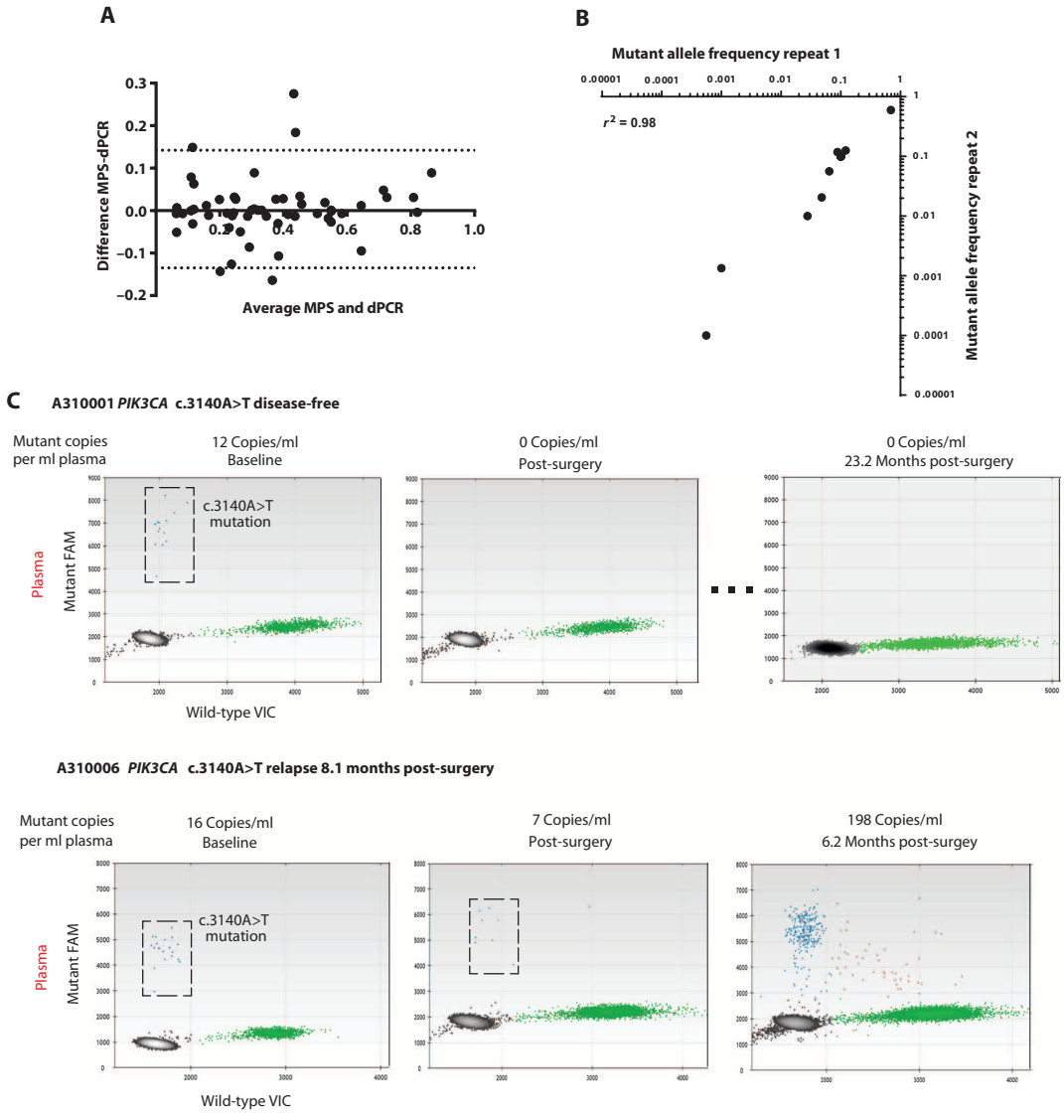

Fig. 2. Personalized, mutation-specific dPCR accurately quantifies ctDNA and is highly reproducible. (**A**) Bland-Altman plot of the agreement between mutational frequency assessed by MPS and by mutation-specific dPCR on baseline tumor DNA, with 95% CI of agreement (–0.13 and 0.14) indicated by dashed lines. Data points from 55 mutation-specific dPCR assays are displayed. (**B**) Correlation of mutation abundance in repeat assays of mutation-specific dPCR assays in plasma DNA (Pearson's correlation coefficient). Data from 17 mutation-specific assays in nine different patients. (**C**) Example of dPCR mutation tracking in two patients with early breast cancer, whose tumors harbored the same tumor *PIK3CA* c.3140A>T (p.H1047L) somatic mutation at the baseline plasma samples. The complete time course for patient A310001 is given in fig. S1. In each dPCR plot, green dots represent WT DNA (VIC-labeled), blue dots represent mutant DNA (FAM-labeled), brown dots represent droplets containing both WT and mutant DNA, and black dots are droplets with no DNA incorporated.

with gliomas frequently have undetectable ctDNA (*9*), suggesting that the detection of recurrences restricted to the brain may be challenging by means of mutation tracking. In contrast, extracranial MRD was predicted by ctDNA mutation tracking in all patients who relapsed (n = 12) (P = 0.0022, χ^2 test with Yates' correction).

Genomic characterization of MRD by high-depth plasma DNA sequencing

Intratumor genetic heterogeneity is found in many solid tumors, including breast cancer (*14*–*16*). Adjuvant therapies targeted at the genetic characteristics of the primary cancer may be ineffective if micrometastatic disease displays different genetic alterations to those found in the primary cancer. Having demonstrated that mutation tracking can anticipate clinical relapse, we set out to establish if high-depth targeted capture MPS of plasma DNA could be used to interrogate the genetic profile of MRD before relapse and, in cancers with intratumor genetic heterogeneity, assess whether the genetic profile of MRD reflected that of the original primary breast cancer or the subsequent metastatic recurrence.

Once mutation tracking had confirmed the presence of MRD, we sequenced DNA from the primary cancer, from the residual primary tumor resected after chemotherapy, from the plasma DNA taken before relapse, and from the subsequent metastasis when biopsied, in five patients with a panel targeting all exons of 273 genes recurrently mutated in breast cancer (*17*). The sequencing strategy was optimized for low DNA inputs using hybrid capture with a custom NimbleGen SeqCap EZ Choice library followed by sequencing on a HiSeq2000 to a mean target depth after duplicate removal of 460× (range, 104× to 1015×). In all five cases, we identified tumor-specific mutations by high-depth sequencing of the plasma DNA taken before clinical relapse, demonstrating its widespread applicability in interrogating the genetics of MRD (Fig. 7 and fig S2). Targeted capture MPS also revealed copy number alterations in the plasma samples of two patients, consistent with those documented in the primary tumor and/ or metastatic relapse (fig. S3). Copy number alterations were not identifiable in the remaining cases, likely because ctDNA was present as a small fraction of total plasma DNA.

We next addressed whether the repertoire of somatic genetic alterations identified in the analysis of ctDNA arising from MRD would merely recapitulate that of the original primary cancer or reveal greater diversity reflective of intratumoral genetic heterogeneity. In one case,

A310006, ctDNA sequencing by MPS revealed no additional genetic events to the *PIK3CA* mutation found in the primary cancer [using AmpliSeq Personal Genome Machine (PGM) because there was insufficient DNA for capture sequencing], suggesting homogeneity in the genetics of the MRD for this patient (fig. S2). However, in all other patients, we uncovered diversity in the genetics of MRD compared to the primary cancer (Fig. 7 and fig S2). For example, plasma DNA sequencing revealed substantial divergence of the genetics of the ctDNA arising from MRD compared to the original primary tumor for patient A310012 (fig. S2).

In patient A310003, sequencing of ctDNA revealed the presence of a *PIK3CA* mutation present in both the primary and metastatic lesion; however, the repertoire of somatic mutations found in the plasma (ctDNA arising from MRD) was more similar to that of the subsequently biopsied metastatic relapse than that of the primary cancer (Fig. 7A). In particular, ctDNA sequencing identified an activating *FGFR1* K656E mutation that was not present in the analyzed primary tumor biopsy but was present in the metastasis (Fig. 7A). The *FGFR1* K656E mutation is directly paralogous to the *FGFR3* K650E activating mutation that is frequently found in bladder cancer and thanatophoric dysplasia type II (*18*, *19*). Similarly, ctDNA sequencing identified loss of an *ESR1* E380Q mutation found in the primary tumor, but not in the metastasis, anticipating loss of ER in the metastasis, which was confirmed by immunohistochemistry (fig. S4A). Sequencing of two foci of residual primary tumor after chemotherapy provided evidence of consistent clonal selection compared to the primary tumor before treatment, although the changes did not predict those that were found in the subsequent metastasis (Fig. 7A). When tracked by dPCR, the primary tumor *PIK3CA* mutation remained present in plasma DNA and metastasis, indicating an early clonal event in the cancer.

In patient A310035, sequencing ctDNA before relapse predicted acquisition of a *SYNE1* S1244Y mutation in the subsequently biopsied metastasis, as well as enrichment for a *GATA3* frameshift mutation and loss of a *STAT3* mutation (Fig. 7B). In this patient, enrichment for the *SYNE1* mutation was demonstrated in the residual tumor after chemotherapy (fig. S4B). Finally, in patient A310004, sequencing of the relapsed tumor revealed an *RB1* R320* somatic mutation that was not detectable by sequencing of the plasma ctDNA taken 8.1 months before relapse (13 months after surgery) (Fig. 7C). We developed and optimized a multiplex dPCR assay (fig. S4C) to track this mutation, along with the other two mutations in *ANK3* and *XIRP2* that were present in both the primary tumor and the metastatic recurrence in this patient. dPCR demonstrated that the *RB1* mutation was a late event, only first detectable in a plasma sample taken 16.1 months after surgery, then expanding in frequency on serial sampling at relapse (Fig. 7C). This suggests that genetic diversity develops in expanding micrometastatic disease before relapse and that mutation tracking may have the potential to identify MRD at a point before genetic diversity develops.

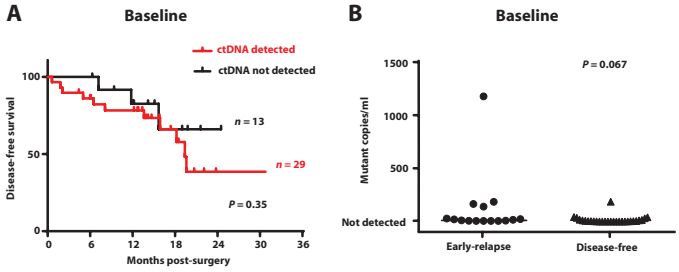

Fig. 3. Early relapse is not predicted by analysis of baseline ctDNA. (**A**) Disease-free survival according to the detection of ctDNA in the baseline plasma sample. *P* value determined by log-rank test. (**B**) Mutant copies per milliliter of plasma at baseline in patients who relapsed early and who did not relapse during follow-up. *P* value determined by Mann-Whitney *U* test. Data in (A) and (B) are from the same *n* = 42 patients. ctDNA associations with other clinicopathological characteristics are in table S2.

DISCUSSION

Here, we show that ctDNA mutation tracking can detect MRD noninvasively and identify earlier which patients are at risk of cancer recurrence. We devised an assay pipeline that uses baseline primary tumor mutations to develop personalized dPCR assays to track

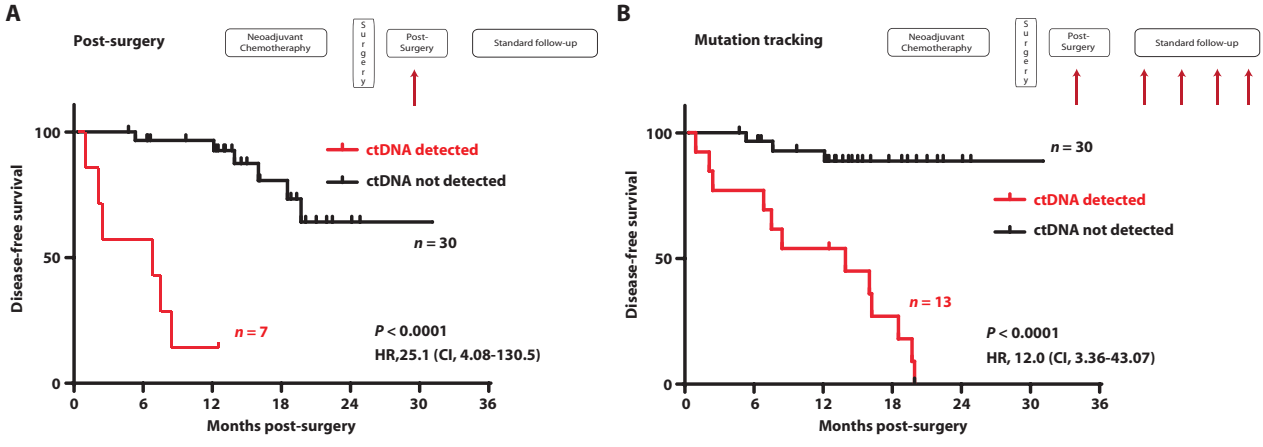

Fig. 4. Mutation tracking in serial plasma samples predicts early relapse. (**A**) Disease-free survival according to the detection of ctDNA in the first postsurgical plasma sample [HR, 25.1 (95% CI, 4.08 to 130.5)]. *P* value determined by log-rank test. Data are from *n* = 37 patients. (**B**) Disease-free survival according to the detection of ctDNA in serial follow-up samples [HR, 12.0 (95% CI, 3.36 to 43.07)]. *P* value determined by log-rank test. Data are from *n* = 43 patients [37 of whom are represented in (A)].

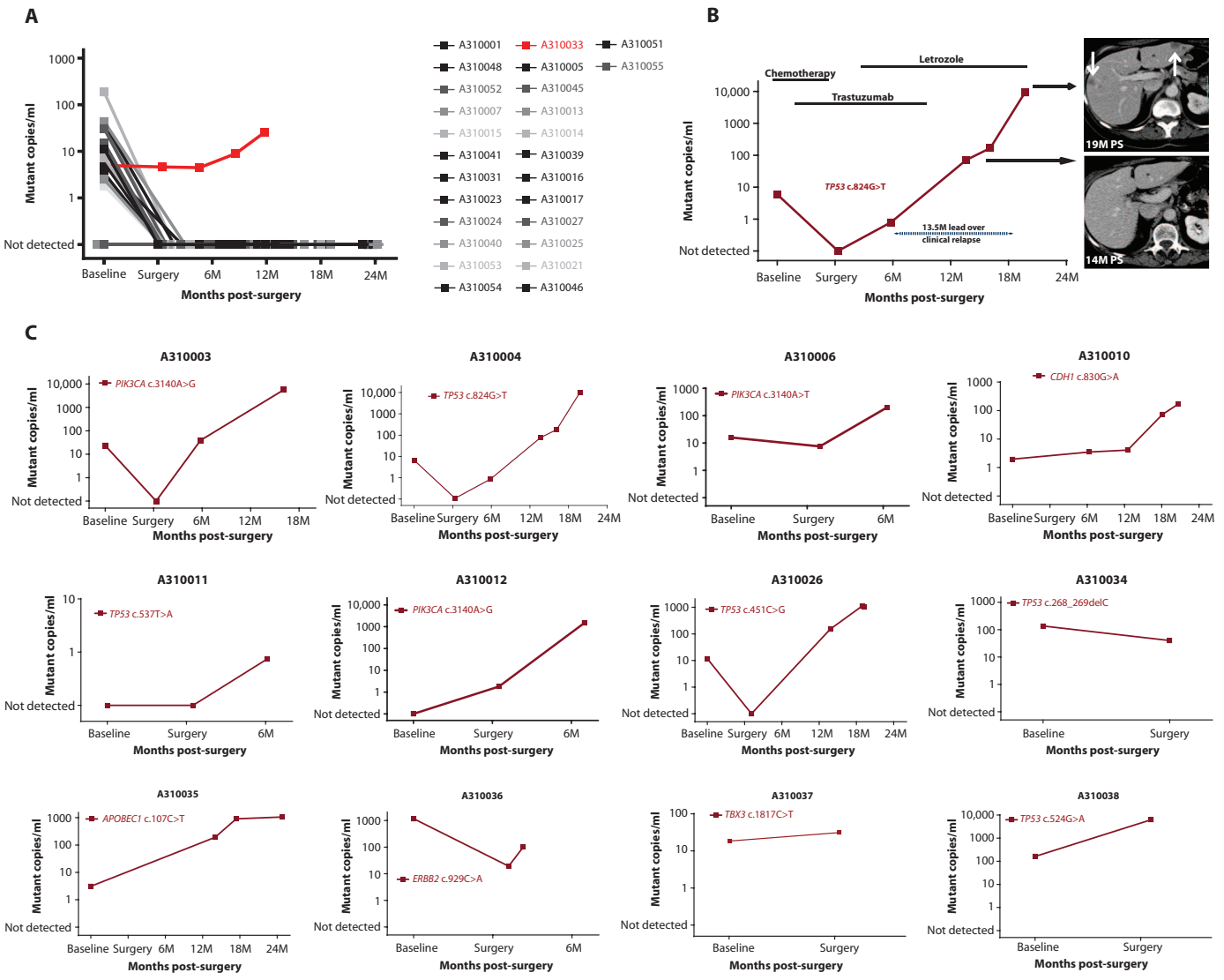

Fig. 5. Mutation tracking in early-relapse and disease-free patients. (**A**) Mutation tracking profile in 26 patients who are currently disease-free after treatment of primary breast cancer. Mutations remain undetectable in the postsurgical and follow-up periods in 25 of 26 patients. The remaining patient (A310033, red), with triple-negative disease, had ctDNA detectable after surgery with an increase in the detectable level of mutational load in follow-up sampling; however, this patient did not have clinical relapse at the time of reporting. (**B**) Mutation tracking detects ctDNA arising from MRD in patient A310004, with a lead time of 13.5 months over clinical relapse. Contrast-enhanced spiral computed tomography (CT) scan revealed no abnormality at 14 months of follow-up, although multiple liver metastases (white arrows) were subsequently detected at 19 months of follow-up. M, months; PS, post-surgery. (**C**) Mutation tracking ctDNA profiles before relapse, from 12 patients who experienced early relapse after treatment of primary breast cancer. After ctDNA was detected in a postsurgical or follow-up sample, it was detected in all subsequent samples before relapse.

the presence of ctDNA in plasma over time. After using dPCR to detect MRD, we showed that high-depth plasma DNA sequencing can help define the repertoire of somatic genetic alterations found in MRD, providing evidence of clonal shifts in response to systemic therapy.

Our data illustrate fundamental principles for the use of ctDNA in the detection of MRD. Driver, and likely clonal, mutations should be tracked in preference to subclonal nondriver mutations, which may be lost in the MRD that subsequently repopulates the metastatic recurrence (A310003, A310035, and A310012). Here, serial sampling during follow-up was required for accurate MRD detection and relapse prediction. Our data suggest that the burden of MRD at a single postsurgical time point soon after completing neoadjuvant chemotherapy is, in some cases, insufficient for its detection in the plasma DNA, or, owing to lack of proliferation and apoptosis in the MRD, there is no release of ctDNA. These findings contrast with a report where ctDNA detection with BEAMing in a single colorectal cancer sample taken after surgical resection of liver metastases offered high predictive potential, potentially because of the high burden of micrometastatic disease in this setting (*10*). Nevertheless, we recommend serial sampling to detect the MRD as it proliferates and expands. Mutation tracking in serial samples may be particularly important in ER-positive

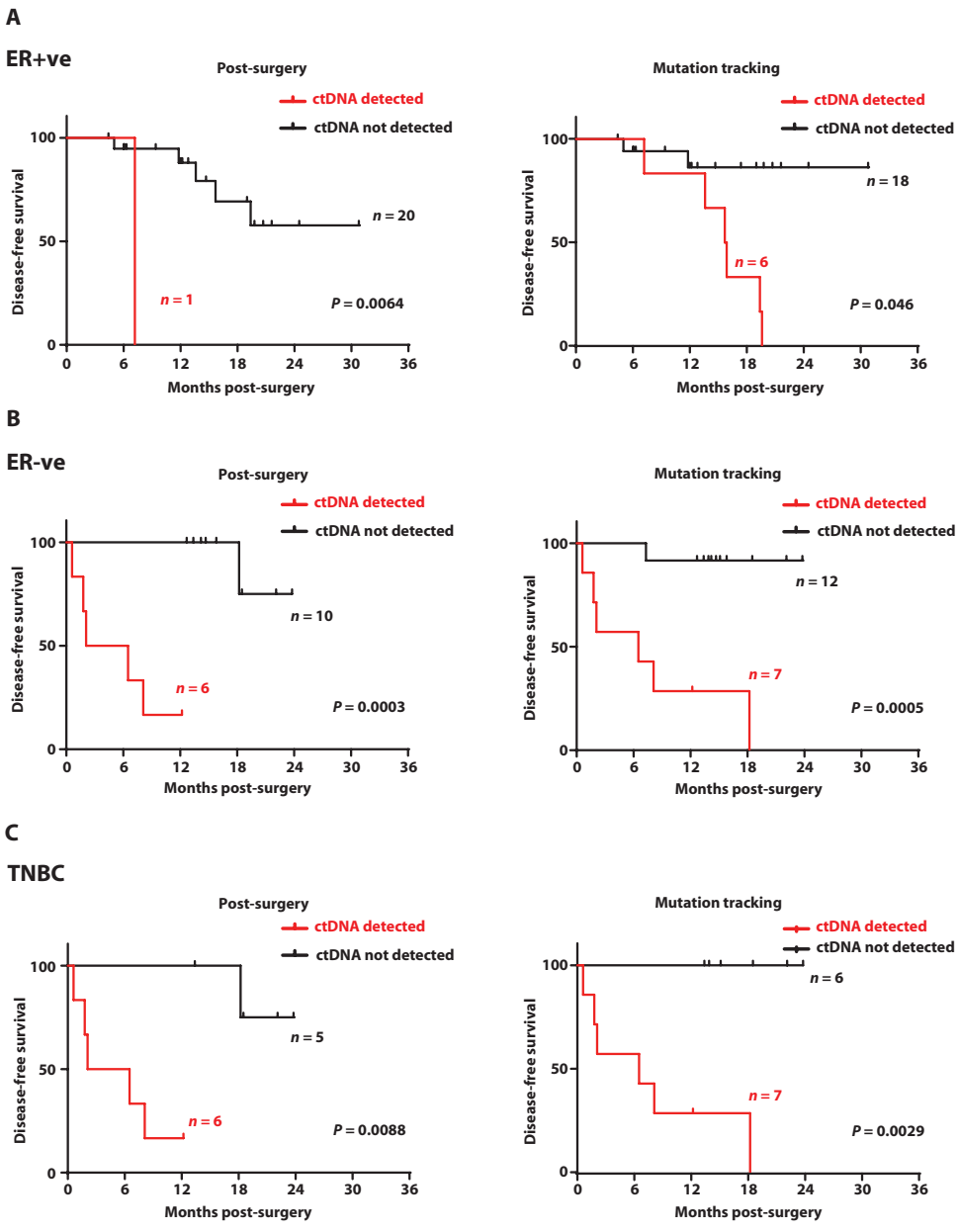

Fig. 6. Disease-free survival prediction based on single post-surgery ctDNA and mutation tracking in serial plasma samples according to tumor subtype. (**A**) ER-positive breast cancers in the first postsurgical sample (n = 21) and serial follow-up samples (n = 24). (**B**) ER-negative breast cancers in the first postsurgical sample (n = 16) and serial follow-up samples (n = 19). (**C**) Triple-negative breast cancers (TNBC) in the first postsurgical sample (n = 11) and serial follow-up samples (n = 13). P values determined by log-rank test.

breast cancer to detect ctDNA changes during postoperative endocrine therapy (Fig. 6).

We used dPCR to assay plasma samples in this study. This relatively cost-efficient technology represents, along with BEAMing, the most sensitive techniques currently available for detection of known rare mutations. Alternative techniques include the detection of structural variants (*11*), although the challenge in advancing this technique to clinical practice is accurate identification of these variants. MPS of plasma is challenged by polymerase error in detecting rare variants, although techniques such as barcoding for error correction (*20*) may allow MPS to challenge dPCR for sensitivity. Nevertheless, no other technologies have been capable of quantifying ctDNA for early detection of MRD.

Intratumor genetic heterogeneity reflecting clonal diversity in cancers (*14*–*16*) presents a potential major barrier for successful adjuvant therapy that aims to eradicate micrometastatic disease and prevent recurrence (*21*). Adjuvant therapies targeted at the genetic characteristics of the primary cancer may be ineffective if micrometastatic disease displays different genetic alterations from those found in the primary cancer. Here, we show that high-depth targeted capture MPS of ctDNA before relapse has the potential to address this challenge by interrogating the genetic characteristics of MRD to identify the lethal clone that may differ in its repertoire of somatic mutations from the dominant clone in the primary cancer. We detected potentially targetable mutations in the ctDNA that were not in the primary tumor; in some cases, other targetable mutations were lost from primary tumor to ctDNA. Resistance mutations can be detected in plasma many months before the development of clinical resistance in the metastatic setting (*22*, *23*), and we demonstrated that this concept can be extended to patients with potential curable micrometastatic disease.

Targeted therapy has the potential to cure patients in the adjuvant setting with micrometastatic cancer (*24*), likely owing to the low disease burden, whereas targeted therapy is almost uniformly unable to cure patients with advanced metastatic disease (*25*). We demonstrate that the micrometastatic disease may evolve over time, with late appearance of an *RB1* mutation that might cause resistance to targeted therapy with cyclin-dependent kinase 4/6 inhibitors. This suggests that the approach described here may lead to the detection of MRD before the establishment of genetic diversity.

Despite our encouraging findings for improving early treatment of breast cancer patients, the follow-up was short (~2 years), with relatively few patients. Sequencing of the plasma DNA taken at relapse, with the capture 273-gene panel, to 752× depth in one patient with a brain metastasis revealed no detectable mutations. The lack of sensitivity for the detection of metastatic disease restricted to the brain likely suggests that the blood-brain barrier blocks the release of ctDNA

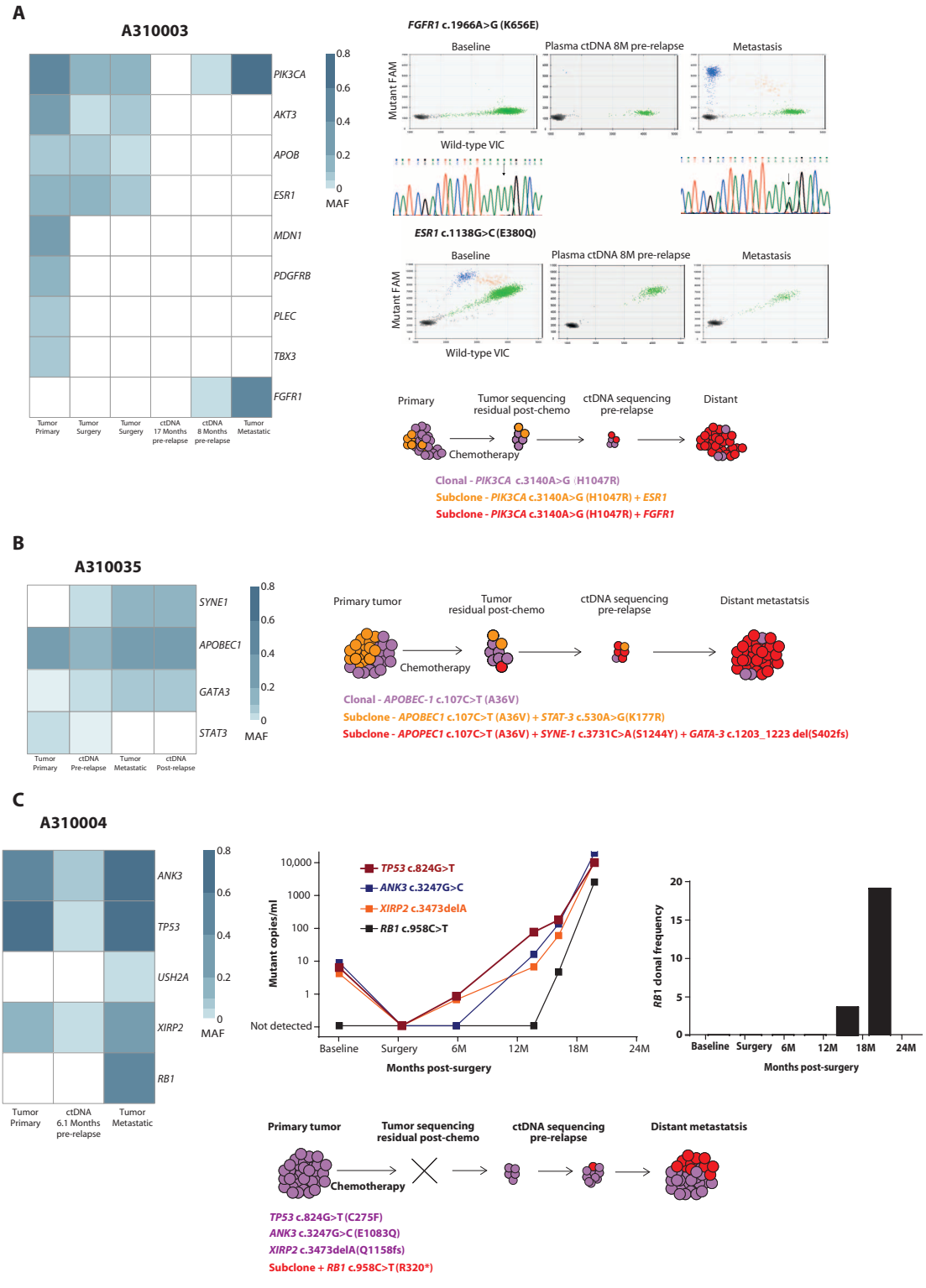

Fig. 7. High-depth targeted capture MPS of plasma DNA to characterize the genomic landscape of MRD. For five patients, DNA was extracted from microdissected primary tumor, residual tumor resected after chemotherapy, metastatic tumor biopsies where available, plasma DNA samples, and germline lymphocyte DNA and was subjected to targeted capture MPS using a custom panel targeting the exons of 273 genes recurrently mutated in breast cancer or involved in DNA repair pathways. Data for two patients are provided in fig. S2. (**A**) (Left) High-depth targeted capture MPS in patient A310003 of baseline primary tumor biopsy, two separate surgery samples after chemotherapy, plasma DNA samples at 17 and 8 months before relapse, and metastatic disease biopsy taken on subsequent relapse. (Right) Validation of MPS findings with dPCR and Sanger sequencing. The *FGFR1* mutation is detectable at a low level 17 months before relapse. A diagram illustrates possible clonal selection in this patient through therapy and time. Immunohistochemistry of baseline and metastatic tumor samples is given in fig. S4A. (**B**) High-depth targeted capture MPS in patient A310035 of baseline primary tumor biopsy, plasma DNA before relapse, relapse biopsy, and plasma DNA after relapse. Analysis of the surgery sample is shown in fig. S4B. (**C**) High-depth targeted capture MPS in patient A310004 of baseline primary tumor biopsy, plasma DNA 6.1 months before relapse, and relapse biopsy (left). Relapse biopsy revealed an *RB1* c.958C>T (R320*) somatic mutation that was not detectable by sequencing of the plasma. (Right) Validation of MPS findings with dPCR. *RB1* mutation was a late arising event that was not detectable 6.1 months before relapse but then rose to relative clonal abundance at relapse ($P < 0.0001$, χ^2 test for trend). There was a pathological complete response to chemotherapy in the primary tumor. MAF, mutant allele frequency.

into circulation. We were unable to address the potential ctDNA detection in patients who had primary surgery and no chemotherapy because our study focused on the ability to detect MRD after neoadjuvant chemotherapy and surgery. In addition, because patients in this series did not have regular imaging studies in the follow-up, a future prospective study comparing with imaging is required. Our results could potentially

be extended by tracking a large number of mutations in each blood sample, in part by greatly extending the targeted sequencing panel to identify multiple mutations in each patient. The theoretical potential for loss of individual driver mutations in metastatic disease could be countered by such an approach, although that is not observed in our series. However, in our study, assaying for three clonal mutations in the postsurgical sample from patient A310004 was unable to detect ctDNA. This suggests that a substantial increase in the number of genetic events tracked per milliliter of plasma would be required.

In conclusion, we have shown that noninvasive mutation tracking in plasma DNA can detect residual MRD, which standard treatment has failed to eradicate, and thus identify patients at high risk of recurrence. Furthermore, we have shown that the genetic events of metastatic disease may differ from those found in the primary tumor. This knowledge will allow for therapeutic interventions tailored to the driver genetic events present in micrometastases in a new approach that could help combat the challenge posed by intratumor genetic heterogeneity.

MATERIALS AND METHODS

Study design

Serial plasma samples were collected from patients with early breast cancer to assess the potential of assays of ctDNA to predict relapse after treatment. Patients (table S5) were recruited from the Royal Marsden Hospital and were treated with standard therapy. Tumor DNA was extracted from the baseline pretreatment tumor biopsy and sequenced with an amplicon PGM library to identify mutations specific to the tumor. dPCR assays specific to that mutation were developed to track the mutation on ctDNA, at baseline, and in sequential plasma samples taken after surgery. The log-rank test was used to assess the association between detection of ctDNA and disease-free survival.

Patient cohort and sample collection

Fifty-five patients scheduled to receive neoadjuvant chemotherapy were analyzed from prospective sample collection studies, the ChemoNEAR study [Research Ethics Committee (REC) ref. no. 11/EE/0063] or the Plasma DNA study (REC ref. no. 10/H0805/50) approved by Research Ethics committees (East of England—Essex and London—Bromley, respectively). Written informed consent was obtained from all participants. Staging investigations were performed at baseline for all node-positive and/or cT3/4 patients with CT scan and bone scans, and those with distant metastatic disease were excluded from the study. Patients were treated with standard neoadjuvant chemotherapy (sequential anthracyclines-taxane–based chemotherapy in 51 patients, sequential anthracyclines–paclitaxel + carboplatin in 2 patients, and docetaxel + cyclophosphamide in 2 patients) with or without trastuzumab depending on HER2 status. After completion of surgery with or without radiotherapy, patients were treated with adjuvant hormone therapy or trastuzumab as per standard local practice and were followed up in a nurse-led open-access follow-up program. In the series, a single patient presented with axillary lymphadenopathy from a cryptic breast primary. This patient had radiotherapy to the breast, axillary, and supraclavicular nodes after neoadjuvant chemotherapy, without surgery.

Core biopsies were taken at baseline, at surgery, and where clinically indicated at recurrence. Plasma samples were collected into EDTA K2 tubes at baseline (before chemotherapy), after surgery (2 to 4 weeks after surgery), and every 6 months during follow-up or until relapse, whichever occurred first (Fig. 1). The clinicopathological characteristics of the study cohort are presented in table S5, whereas a CONSORT (Consolidated Standards of Reporting Trials) flow diagram of the patients included in the study is presented in fig. S5. ER, progesterone receptor (PR), and HER2 status were assessed in a single laboratory at the Royal Marsden Histopathology Department using standard criteria.

Processing and DNA extraction from tumor samples

Tissues from core biopsies taken at diagnosis, surgery, and recurrence were formalin-fixed and paraffin-embedded (FFPE). Four to eight sections (4 μm thick) were stained with Nuclear Fast Red and were microdissected under a stereomicroscope to achieve >70% tumor cell content. Tumor DNA was isolated using the QIAamp DNA FFPE Tissue Kit (Qiagen) as per the manufacturer's instructions. Germline DNA was extracted from buffy coat DNA using the DNeasy Blood and Tissue Kit (Qiagen) as per the manufacturer's instructions.

Processing of plasma and extraction of circulating DNA

Blood collected in EDTA K2 tubes was processed within 2 hours of sample collection and centrifuged at 1600 rpm for 20 min, with plasma stored at −80°C until DNA extraction. DNA was extracted from 2 to 4 ml of plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer's instructions. The DNA was eluted into 50 μl of AVE buffer and stored at −20°C.

DNA quantifications from tissue and/or plasma

DNA isolated from tissue or plasma was quantified on a Bio-Rad QX100 ddPCR using ribonuclease P (RNase P) as the reference gene. The eluate (1 μl) was added to a dPCR reaction containing 10 μl of ddPCR Supermix for Probes (Bio-Rad) and 1 μl of TaqMan Copy Number Reference Assay, human, RNase P (Life Technologies) on a total volume of 20 μl. The reaction was partitioned into ~14,000 droplets per sample in a QX100 droplet generator according to the manufacturer's instructions. Emulsified PCR reactions were run on 96-well plates on a G-Storm GS4 thermal cycler, incubating the plates at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s, then by a 10-min incubation at 98°C. The temperature ramp increment was 2.5°C/s for all steps. The plates were read on a Bio-Rad QX100 droplet reader using QuantaSoft version 1.4.0.99 software from Bio-Rad. At least two negative control wells with no DNA were included in every run. The amount of amplifiable RNase P DNA was calculated using the Poisson distribution in QuantaSoft.

Assessment of recovery of mutant DNA extracted from plasma

Genomic DNA was extracted from the *PIK3CA* mutation c.3140A>T GP2d colon adenocarcinoma cell line (European Collection of Cell Cultures cat. no. 95090714), using the DNeasy Blood and Tissue Kit (Qiagen) as per the manufacturer's instructions. DNA was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies) as per the manufacturer's instructions. Genomic DNA (5 μg) was restriction-digested using Hind III endonuclease, and the concentration of c.3140A>T mutant DNA copies was calculated using a dPCR assay on at least five replicates on the QX100 ddPCR system (Bio-Rad) using the *PIK3CA* c.3140A>T primers and probes described in table S1. A total of 150 copies of the *PIK3CA* c.3140A>T mutation were spiked into 1 ml of WT plasma samples and immediately processed as described above. Of the input *PIK3CA* mutant DNA, 43% (95% CI, ±2.2%) was recovered and analyzed by the dPCR assay.

Ion PGM sequencing of baseline tumor samples

Sequencing libraries were prepared with a custom Ion AmpliSeq Breast Cancer Panel targeting 14 known breast cancer driver genes (*26*) (table S6) using the Ion AmpliSeq Library Preparation protocol with 5 ng of tumor DNA, according to the manufacturer's instructions. After barcoding, the libraries were quantified using quantitative PCR, diluted to 100 pM, and pooled. The libraries were templated with the Ion PGM Template OT2 200 Kit (Life Technologies) and sequenced on a 318 PGM chip using Ion PGM Sequencing 200 Kit v2 (Life Technologies) and 500 flows to a mean depth of ×2355. The sequencing resulted in 200,000 to 650,000 reads per sample. Variant Caller v4.0.r73742 with no HotSpot region and on "Germ-Line–Low Stringency" configuration was used for calling variants, and variants not reported as germ line in dbSNP (Single Nucleotide Polymorphism database) were selected as potentially being somatic mutations. Potential somatic mutations were cross-referenced against the 1000 Genomes Project database (www.1000genomes.org), and only variants that did not appear on the 1000 Genomes Project database were taken forward for development of dPCR assays.

Hybrid capture MPS

In five patients who experienced relapse, DNA extracted from microdissected primary tumor, residual tumor resected after chemotherapy, metastatic tumor biopsies where available, plasma DNA samples, and germline lymphocyte DNA to exclude germline polymorphisms was subjected to targeted capture MPS. Custom oligonucleotides (Roche NimbleGen SeqCap EZ Choice) were designed for hybridization capture of all protein-coding exons of 273 genes recurrently mutated in breast cancer or involved in DNA repair pathways, as previously described (*17*, *27*). Barcoded sequence libraries were prepared (NEXTflex barcode adapters, Bioo Scientific) using 12.5 to 50 ng of DNA and amplified (KAPA Biosystems), and 12 barcoded libraries were pooled at equimolar concentrations into a single exon capture reaction, as previously described (*17*, *27*, *28*). Paired-end 75 × 75–base pair sequencing was performed on a single lane of an Illumina HiSeq2000 flow cell. Sequencing reads were aligned to the reference human genome hg19 using the Burrows-Wheeler Aligner (version 0.6.2) (*29*), and local realignment and base quality recalibration were performed using the Genome Analysis Toolkit (GATK) (*30*). Duplicates were removed using SAMtools (*31*). Somatic single nucleotide variants (SNVs) were identified using MuTect (*32*), GATK Haplotype Caller (version 3.1.1) (*30*), and Strelka (*33*). To minimize potential false-positive results obtained with high-depth targeted MPS performed with DNA extracted from FFPE tissues or plasma, only SNVs identified by at least two of the three callers used were considered valid, as previously described (*17*). Small insertions and deletions (indels) were identified using GATK Haplotype Caller and VarScan2 (version 2.3.6) (*34*), gene copy number aberrations were generated using VarScan2 and segmented using circular binary segmentation, and gains and losses were called using the R package CGHcall. All candidate mutations identified in a given tumor or plasma DNA sample were validated manually in all other plasma DNA or tumor samples of the given patient, and mutations supported by two or more reads were regarded as being present (table S7).

Development of mutation-specific dPCR assays

dPCR was performed on a QX100 ddPCR system (Bio-Rad) using TaqMan chemistry. For each tumor mutation, we designed a primer probe combination using Primer 3 Plus or Life Technologies' custom SNP genotyping assays tool (table S1). Primers and probes were designed to avoid reported SNPs. Primers and probes were analyzed for the presence of hairpins, secondary structures, or hetero/homodimer formation. Primers were analyzed for specificity using the electronic PCR (ePCR) tool of the University of California, Santa Cruz (http:// genome.ucsc.edu/cgi-bin/hgPcr?command=start). dPCR conditions were optimized with a temperature gradient to identify the optimal annealing temperature using either a WT DNA spiked with a mutant synthetic oligonucleotide or cell line DNA known to carry the mutation. After optimization, the baseline tumor DNA sample was analyzed to validate the assay. The complete absence of mutation in the corresponding patient's germline lymphocyte DNA, as well as unmatched plasma samples from metastatic breast cancer patients, was confirmed by dPCR.

dPCR analysis of circulating free plasma DNA

dPCR was performed on a QX100 ddPCR system (Bio-Rad) using TaqMan chemistry with primers and probes described in table S1 at a final concentration of 900 nM primers and 250 nM probes. PCR reactions were prepared with ddPCR Supermix for Probes (Bio-Rad) and partitioned into a median of 50,000 droplets per sample in a QX100 droplet generator according to the manufacturer's instructions. DNA extracted from 4 ml of plasma was analyzed for the presence of the mutation at each time point. Emulsified PCR reactions were run on a 96-well plate on a G-Storm GS4 thermal cycler, incubating the plates at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and specific assay extension temperature (table S1) for 60 s, then by a 10-min incubation at 98°C. The temperature ramp increment was 2.5°C/s for all steps. Plates were read on a Bio-Rad QX100 droplet reader using QuantaSoft version 1.4.0.99 software from Bio-Rad to assess the number of droplets positive for mutant DNA, WT DNA, both, or neither. At least two negative control wells with no DNA were included in every run.

dPCR analysis

To assess mutation fraction, the concentration of mutant DNA (copies of mutant DNA per droplet) was estimated from the Poisson distribution: number of mutant copies per droplet (Mmu) = $-\ln[1 - (\text{nmu}/n)]$, where nmu is the number of droplets positive for mutant FAM probe and n is the total number of droplets. The DNA concentration in the reaction was estimated as follows: MDNAconc = $-\ln[1 - (\text{nDNAconc}/n)]$, where nDNAconc is the number of droplets positive for mutant FAM probe and/or WT VIC probe and n is the total number of droplets. The fraction mutation was computed as follows: fraction mutation = Mmu/MDNAconc. To assess the number of mutant copies per milliliter of plasma, the number of mutant FAM–positive droplets was adjusted for the number of wells run for the sample, the total number of droplets generated, the median volume of a droplet (0.89 pl), and the volume equivalent of plasma run, using the following formula:

$$\text{Mutant copies per milliliter} = (\text{total number of droplets positive for FAM}) \times 20,000 \times \text{number of wells run} / \text{volume of plasma equivalents}) / (\text{total number of droplets generated} \times 0.89)$$

A mutation was only considered to be present if two or more FAM-positive droplets were detected in 4 ml of plasma equivalent DNA, with this criterion for a positive test being predefined. After taking into account the recovery of 43% of DNA present in plasma (as calculated above), the dPCR assay would be anticipated to detect tumor-specific mutations with 86% probability at an actual concentration of 2 mutations/ml and 99% probability at an actual concentration of 4 mutations/ml.

The presence of increasing ctDNA abundance was defined as the doubling of mutant copies per milliliter from the nadir or the appearance of ctDNA when previously undetectable. Detection in baseline or after surgery was defined as detection of ctDNA at a single time point. Mutation tracking was defined as detection of ctDNA in any of the postsurgical and serial ctDNA samples.

Statistical analysis

The primary endpoint of the study was to assess disease-free survival in patients with and without detection of ctDNA using univariable survival estimates calculated using the Kaplan-Meier method, and survival differences were estimated using the log-rank test. Multivariable Cox regression analyses were used to test the independent prognostic value of mutation tracking and ctDNA, adjusted for tumor size, pathological nodal status, and molecular subtypes. Harrell's C-index from the univariable and multivariable survival model was calculated (*35*). The C-index is a probability of concordance between predicted and observed survival, defined as the probability that risk assignments to members of a random pair are accurately ranked according to their prognosis. A C-index of 0.5 indicates random prediction, and higher values indicate increasing prediction accuracy. The associations of median ctDNA level with clinicopathological markers were assessed using the Mann-Whitney *U* or the Krustal-Wallis test where appropriate. The odds ratios of ctDNA detection with the standard clinicopathological variables were estimated using univariable logistic regression analysis. All statistical analyses were performed with GraphPad Prism version 6.0 or in R 3.0.1 with R packages *survival*, *Hmisc*, and *rms*. All *P* values are two-sided. Disease-free survival, excluding contralateral invasive breast cancers, was assessed from the date of surgery.

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/7/302/302ra133/DC1
Fig. S1. Mutation tracking by dPCR along a 24-month follow-up of a disease-free patient.
Fig. S2. High-depth targeted capture MPS on plasma DNA from two relapsed patients.
Fig. S3. Copy number profile in primary tumor and plasma DNA before relapse.
Fig. S4. Validation and follow-up of MPS on plasma DNA.
Fig. S5. CONSORT flow diagram of patients included in this study.
Table S1. dPCR assays and mutations analyzed in this study.
Table S2. Clinicopathological factors associated with baseline ctDNA level.
Table S3. Prediction of disease-free survival using a single postsurgical blood sample.
Table S4. Prediction of disease-free survival by mutation tracking using serial blood samples.
Table S5. Summary of the study cohort.
Table S6. Ion AmpliSeq breast cancer driver gene panel.
Table S7. Reads from capture MPS of tumor and plasma DNA.

REFERENCES AND NOTES

1. A. Jemal, F. Bray, M. M. Center, J. Ferlay, E. Ward, D. Forman, Global cancer statistics. *CA Cancer J. Clin.* **61**, 69–90 (2011).
2. N. Howlader, S. F. Altekruse, C. I. Li, V. W. Chen, C. A. Clarke, L. A. G. Ries, K. A. Cronin, US incidence of breast cancer subtypes defined by joint hormone receptor and HER2 status. *J. Natl. Cancer Inst.* **106**, dju055 (2014).
3. S. A. Leon, B. Shapiro, D. M. Sklaroff, M. J. Yaros, Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res.* **37**, 646–650 (1977).
4. T. Forshew, M. Murtaza, C. Parkinson, D. Gale, D. W. Y. Tsui, F. Kaper, S.-J. Dawson, A. M. Piskorz, M. Jimenez-Linan, D. Bentley, J. Hadfield, A. P. May, C. Caldas, J. D. Brenton, N. Rosenfeld, Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. *Sci. Transl. Med.* **4**, 136ra168 (2012).
5. S.-J. Dawson, D. W. Y. Tsui, M. Murtaza, H. Biggs, O. M. Rueda, S.-F. Chin, M. J. Dunning, D. Gale, T. Forshew, B. Mahler-Araujo, S. Rajan, S. Humphray, J. Becq, D. Halsall, M. Wallis, D. Bentley, C. Caldas, N. Rosenfeld, Analysis of circulating tumor DNA to monitor metastatic breast cancer. *N. Engl. J. Med.* **368**, 1199–1209 (2013).
6. M. Murtaza, S.-J. Dawson, D. W. Y. Tsui, D. Gale, T. Forshew, A. M. Piskorz, C. Parkinson, S.-F. Chin, Z. Kingsbury, A. S. C. Wong, F. Marass, S. Humphray, J. Hadfield, D. Bentley, T. M. Chin, J. D. Brenton, C. Caldas, N. Rosenfeld, Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. *Nature* **497**, 108–112 (2013).
7. H. Gevensleben, I. Garcia-Murillas, M. K. Graeser, G. Schiavon, P. Osin, M. Parton, I. E. Smith, A. Ashworth, N. C. Turner, Noninvasive detection of *HER2* amplification with plasma DNA digital PCR. *Clin. Cancer Res.* **19**, 3276–3284 (2013).
8. J. A. Beaver, D. Jelovac, S. Balukrishna, R. L. Cochran, S. Croessmann, D. J. Zabransky, H. Yuen Wong, P. Valda Toro, J. Cidado, B. G. Blair, D. Chu, T. Burns, M. J. Higgins, V. Stearns, L. Jacobs, M. Habibi, J. Lange, P. J. Hurley, J. Lauring, D. A. VanDenBerg, J. Kessler, S. Jeter, M. L. Samuels, D. Maar, L. Cope, A. Cimino-Mathews, P. Argani, A. C. Wolff, B. Ho Park, Detection of cancer DNA in plasma of patients with early-stage breast cancer. *Clin. Cancer Res.* **20**, 2643–2650 (2014).
9. C. Bettegowda, M. Sausen, R. J. Leary, I. Kinde, Y. Wang, N. Agrawal, B. R. Bartlett, H. Wang, B. Luber, R. M. Alani, E. S. Antonarakis, N. S. Azad, A. Bardelli, H. Brem, J. L. Cameron, C. C. Lee, L. A. Fecher, G. L. Gallia, P. Gibbs, D. Le, R. L. Giuntoli, M. Goggins, M. D. Hogarty, M. Holdhoff, S.-M. Hong, Y. Jiao, H. H. Juhl, J. J. Kim, G. Siravegna, D. A. Laheru, C. Lauricella, M. Lim, E. J. Lipson, S. K. N. Marie, G. J. Netto, K. S. Oliner, A. Olivi, L. Olsson, G. J. Riggins, A. Sartore-Bianchi, K. Schmidt, I.-M. Shih, S. M. Oba-Shinjo, S. Siena, D. Theodorescu, J. Tie, T. T. Harkins, S. Veronese, T.-L. Wang, J. D. Weingart, C. L. Wolfgang, L. D. Wood, D. Xing, R. H. Hruban, J. Wu, P. J. Allen, C. M. Schmidt, M. A. Choti, V. E. Velculescu, K. W. Kinzler, B. Vogelstein, N. Papadopoulos, L. A. Diaz Jr., Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci. Transl. Med.* **6**, 224ra224 (2014).
10. F. Diehl, K. Schmidt, M. A. Choti, K. Romans, S. Goodman, M. Li, K. Thornton, N. Agrawal, L. Sokoll, S. A. Szabo, K. W. Kinzler, B. Vogelstein, L. A. Diaz Jr., Circulating mutant DNA to assess tumor dynamics. *Nat. Med.* **14**, 985–990 (2008).
11. T. Reinert, L. V. Schøler, R. Thomsen, H. Tobiasen, S. Vang, I. Nordentoft, P. Lamy, A.-S. Kannerup, F. V. Mortensen, K. Stribolt, S. Hamilton-Dutoit, H. J. Nielsen, S. Laurberg, N. Pallisgaard, J. S. Pedersen, T. F. Ørntoft, C. L. Andersen, Analysis of circulating tumour DNA to monitor disease burden following colorectal cancer surgery. *Gut* 10.1136/gutjnl-2014-308859 (2015).
12. B. Vogelstein, K. W. Kinzler, Digital PCR. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 9236–9241 (1999).
13. B. J. Hindson, K. D. Ness, D. A. Masquelier, P. Belgrader, N. J. Heredia, A. J. Makarewicz, I. J. Bright, M. Y. Lucero, A. L. Hiddessen, T. C. Legler, T. K. Kitano, M. R. Hodel, J. F. Petersen, P. W. Wyatt, E. R. Steenblock, P. H. Shah, L. J. Bousse, C. B. Troup, J. C. Mellen, D. K. Wittmann, N. G. Erndt, T. H. Cauley, R. T. Koehler, A. P. So, S. Dube, K. A. Rose, L. Montesclaros, S. Wang, D. P. Stumbo, S. P. Hodges, S. Romine, F. P. Milanovich, H. E. White, J. F. Regan, G. A. Karlin-Neumann, C. M. Hindson, S. Saxonov, B. W. Colston, High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Anal. Chem.* **83**, 8604–8610 (2011).
14. S. P. Shah, A. Roth, R. Goya, A. Oloumi, G. Ha, Y. Zhao, G. Turashvili, J. Ding, K. Tse, G. Haffari, A. Bashashati, L. M. Prentice, J. Khattra, A. Burleigh, D. Yap, V. Bernard, A. McPherson, K. Shumansky, A. Crisan, R. Giuliany, A. Heravi-Moussavi, J. Rosner, D. Lai, I. Birol, R. Varhol, A. Tam, N. Dhalla, T. Zeng, K. Ma, S. Chan, M. Griffith, A. Moradian, S.-W. Grace Cheng, G. B. Morin, P. Watson, K. Gelmon, S. Chia, S.-F. Chin, C. Curtis, O. Rueda, P. D. Pharoah, S. Damaraju, J. Mackey, K. Hoon, T. Harkins, V. Tadigotla, M. Sigaroudinia, P. Gascard, T. Tlsty, J. F. Costello, I. M. Meyer, C. J. Eaves, W. W. Wasserman, S. Jones, D. Huntsman, M. Hirst, C. Caldas, M. A. Marra, S. Aparicio, The clonal and mutational evolution spectrum of primary triple-negative breast cancers. *Nature* **486**, 395–399 (2012).
15. N. Navin, J. Kendall, J. Troge, P. Andrews, L. Rodgers, J. McIndoo, K. Cook, A. Stepansky, D. Levy, D. Esposito, L. Muthuswamy, A. Krasnitz, R. McCombie, J. Hicks, M. Wigler, Tumour evolution inferred by single cell sequencing. *Nature* **472**, 90–94 (2011).
16. S. Nik-Zainal, L. P. Van, D. C. Wedge, L. B. Alexandrov, C. D. Greenman, K. W. Lau, K. Raine, D. Jones, J. Marshall, M. Ramakrishna, A. Shlien, S. L. Cooke, J. Hinton, A. Menzies, L. A. Stebbings, C. Leroy, M. Jia, R. Rance, L. J. Mudie, S. J. Gamble, P. J. Stephens, S. McLaren, P. S. Tarpey, E. Papaemmanuil, H. R. Davies, I. Varela, D. J. McBride, G. R. Bignell, K. Leung, A. P. Butler, J. W. Teague, S. Martin, G. Jönsson, O. Mariani, S. Boyault, P. Miron, A. Fatima, A. Langerød, S. A. J. R. Aparicio, A. Tutt, A. M. Sieuwerts, Å. Borg, G. Thomas, A. V. Salomon, A.-L. Richardson, A.-L. Børresen-Dale, P. A. Futreal, M. R. Stratton, P. J. Campbell; Breast Cancer Working Group of the International Cancer Genome Consortium, The life history of 21 breast cancers. *Cell* **149**, 994–1007 (2012).
17. R. Natrajan, P. M. Wilkerson, C. Marchiò, S. Piscuoglio, C. K. Y. Ng, P. Wai, M. B. Lambros, E. P. Samartzis, K. J. Dedes, J. Frankum, I. Bajrami, A. Kopec, A. Mackay, R. A'hern, K. Fenwick, I. Kozarewa, J. Hakas, C. Mitsopoulos, D. Hardisson, C. J. Lord, C. Kumar-Sinha, A. Ashworth, B. Weigelt, A. Sapino, A. M. Chinnaiyan, C. A. Maher, J. S. Reis-Filho, Characterization of the genomic features and expressed fusion genes in micropapillary carcinomas of the breast. *J. Pathol.* **232**, 553–565 (2014).
18. P. M. Lievens, E. Liboi, The thanatophoric dysplasia type II mutation hampers complete maturation of fibroblast growth factor receptor 3 (FGFR3), which activates signal transducer and activator of transcription 1 (STAT1) from the endoplasmic reticulum. *J. Biol. Chem.* **278**, 17344–17349 (2003).

19. D. Cappellen, C. De Oliveira, D. Ricol, S. de Medina, J. Bourdin, X. Sastre-Garau, D. Chopin, J. P. Thiery, F. Radvanyi, Frequent activating mutations of FGFR3 in human bladder and cervix carcinomas. *Nat. Genet.* **23**, 18–20 (1999).
20. I. Kinde, J. Wu, N. Papadopoulos, K. W. Kinzler, B. Vogelstein, Detection and quantification of rare mutations with massively parallel sequencing. *Proc. Natl. Acad. Sci. U S A.* **108**, 9530–9535 (2011).
21. N. C. Turner, J. S. Reis-Filho, Genetic heterogeneity and cancer drug resistance. *Lancet Oncol.* **13**, e178–e185 (2012).
22. S. Misale, R. Yaeger, S. Hobor, E. Scala, M. Janakiraman, D. Liska, E. Valtorta, R. Schiavo, M. Buscarino, G. Siravegna, K. Bencardino, A. Cercek, C.-T. Chen, S. Veronese, C. Zanon, A. Sartore-Bianchi, M. Gambacorta, M. Gallicchio, E. Vakiani, V. Boscaro, E. Medico, M. Weiser, S. Siena, F. Di Nicolantonio, D. Solit, A. Bardelli, Emergence of *KRAS* mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. *Nature* **486**, 532–536 (2012).
23. L. A. Diaz Jr., R. T. Williams, J. Wu, I. Kinde, J. R. Hecht, J. Berlin, B. Allen, I. Bozic, J. G. Reiter, M. A. Nowak, K. W. Kinzler, K. S. Oliner, B. Vogelstein, The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. *Nature* **486**, 537–540 (2012).
24. D. Slamon, W. Eiermann, N. Robert, T. Pienkowski, M. Martin, M. Press, J. Mackey, J. Glaspy, A. Chan, M. Pawlicki, T. Pinter, V. Valero, M.-C. Liu, G. Sauter, G. von Minckwitz, F. Visco, V. Bee, M. Buyse, B. Bendahmane, I. Tabah-Fisch, M.-A. Lindsay, A. Riva, J. Crown; Breast Cancer International Research Group, Adjuvant trastuzumab in HER2-positive breast cancer. *N. Engl. J. Med.* **365**, 1273–1283 (2011).
25. D. J. Slamon, B. Leyland-Jones, S. Shak, H. Fuchs, V. Paton, A. Bajamonde, T. Fleming, W. Eiermann, J. Wolter, M. Pegram, J. Baselga, L. Norton, Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N. Engl. J. Med.* **344**, 783–792 (2001).
26. V. Taly, D. Pekin, A. El Abed, P. Laurent-Puig, Detecting biomarkers with microdroplet technology. *Trends Mol. Med.* **18**, 405–416 (2012).
27. H. H. Won, S. N. Scott, A. R. Brannon, R. H. Shah, M. F. Berger, Detecting somatic genetic alterations in tumor specimens by exon capture and massively parallel sequencing. *J. Vis. Exp.*, e50710 (2013).
28. L. De Mattos-Arruda, F.-C. Bidard, H. H. Won, J. Cortes, C. K. Ng, V. Peg, P. Nuciforo, A. A. Jungbluth, B. Weigelt, M. F. Berger, J. Seoane, J. S. Reis-Filho, Establishing the origin of metastatic deposits in the setting of multiple primary malignancies: The role of massively parallel sequencing. *Mol. Oncol.* **8**, 150–158 (2014).
29. S. Dawood, K. Broglio, A. U. Buzdar, G. N. Hortobagyi, S. H. Giordano, Prognosis of women with metastatic breast cancer by *HER2* status and trastuzumab treatment: An institutional-based review. *J. Clin. Oncol.* **28**, 92–98 (2010).
30. A. McKenna, M. Hanna, E. Banks, A. Sivachenko, K. Cibulskis, A. Kernytsky, K. Garimella, D. Altshuler, S. Gabriel, M. Daly, M. A. DePristo, The Genome Analysis Toolkit: A MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res.* **20**, 1297–1303 (2010).
31. H. Li, B. Handsaker, A. Wysoker, T. Fennell, J. Ruan, N. Homer, G. Marth, G. Abecasis, R. Durbin; 1000 Genome Project Data Processing Subgroup, The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**, 2078–2079 (2009).
32. K. Cibulskis, M. S. Lawrence, S. L. Carter, A. Sivachenko, D. Jaffe, C. Sougnez, S. Gabriel, M. Meyerson, E. S. Lander, G. Getz, Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. *Nat. Biotechnol.* **31**, 213–219 (2013).
33. C. T. Saunders, W. S. W. Wong, S. Swamy, J. Becq, L. J. Murray, R. Keira Cheetham, Strelka: Accurate somatic small-variant calling from sequenced tumor–normal sample pairs. *Bioinformatics* **28**, 1811–1817 (2012).
34. D. C. Koboldt, Q. Zhang, D. E. Larson, D. Shen, M. D. McLellan, L. Lin, C. A. Miller, E. R. Mardis, L. Ding, R. K. Wilson, VarScan 2: Somatic mutation and copy number alteration discovery in cancer by exome sequencing. *Genome Res.* **22**, 568–576 (2012).
35. F. E. Harrell Jr., K. L. Lee, D. B. Mark, Multivariable prognostic models: Issues in developing models, evaluating assumptions and adequacy, and measuring and reducing errors. *Stat. Med.* **15**, 361–387 (1996).

Acknowledgments: We thank N. Orr, K. Tomczyk, D. Novo, E. Folkerd, M. Afentakis, K. Sidhu, and F. Daley for technical assistance; the Breast Unit Research Team at Royal Marsden Hospital for support in the enrollment of patients and sample collection. **Funding:** NIHR funding to the Royal Marsden Biomedical Research Centre, Breast Cancer Now with generous support from the Mary-Jean Mitchell Green Foundation, Cancer Research UK C30746/A16642, the Cridlan Trust and the Breast Cancer Research Foundation. **Author contributions:** N.C.T., I.E.S., and M.D. designed the trial. N.C.T., I.E.S., and G.S. contributed to the recruitment of patients. I.G.-M., G.S., and S.H. extracted DNA and performed sequencing and dPCR experiments. P.O. and A.N. performed pathology and microdissection. B.W. and I.K. performed MPS. C.N., R.J.C., M.C., and J.A.G. were responsible for bioinformatic analyses. I.G.-M., G.S., J.S.R.-F., and N.C.T. analyzed and interpreted the data. All authors contributed to the writing or review of the manuscript. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** Targeted capture sequencing data are deposited in the National Center for Biotechnology Information (NCBI) biosample database with accession number SRP058761.

Submitted 2 March 2015
Accepted 8 July 2015
Published 26 August 2015
10.1126/scitranslmed.aab0021

Citation: I. Garcia-Murillas, G. Schiavon, B. Weigelt, C. Ng, S. Hrebien, R. J. Cutts, M. Cheang, P. Osin, A. Nerurkar, I. Kozarewa, J. A. Garrido, M. Dowsett, J. S. Reis-Filho, I. E. Smith, N. C. Turner, Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer. *Sci. Transl. Med.* **7**, 302ra133 (2015).

Science Translational Medicine
AAAS

Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer

Isaac Garcia-Murillas *et al.*

Sci Transl Med **7**, 302ra133 (2015);
DOI: 10.1126/scitranslmed.aab0021

Editor's Summary

Risk of recurrence

Predicting whether a cancer patient will relapse remains a formidable challenge in modern medicine. Fortunately, circulating tumor DNA (ctDNA) present in the blood may give clues on residual disease—cancer cells left behind to seed new tumors even after treatment. Garcia-Murillas *et al.* developed a personalized ctDNA assay based on digital polymerase chain reaction to track mutations over time in patients with early-stage breast cancer who had r eceived apparently curative treatments, surgery, and chemotherapy. Mutation tracking in serial samples accurately predicted metastatic relapse—in several instances, months before clinical relapse (median of ~8 months). Such unprecedented early prediction could allow for intervention before the reappearance of cancer in high-risk patients. In addition, the authors were able to shed light on the genetic events driving such metastases, by massively parallel sequencing of the ctDNA, which could inform new drug-based therapies on the basis of the patients' individual mutations.

Science Translational Medicine (print ISSN 1946-6234; online ISSN 1946-6242) is published weekly, except the last week in December, by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005.