

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

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Analysis of Circulating Tumor DNA to Monitor Metastatic Breast Cancer

Sarah-Jane Dawson*, Dana W.Y. Tsui*, Muhammed Murtaza, Heather Biggs, Oscar M. Rueda, Suet-Feung Chin, Mark J. Dunning, Davina Gale, Tim Forshaw, Betania Mahler-Araujo, Sabrina Rajan, Sean Humphray, Jennifer Becq, David Halsall, Matthew Wallis, David Bentley, Carlos Caldas[@] and Nitzan Rosenfeld[@]

Supplementary Appendix

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Supplementary Methods

Subjects and sample collection

We conducted a prospective clinical study (DETECT: Identification and classification of circulating tumor cells and circulating nucleic acids in patients with metastatic breast cancer) at a single centre within the UK (Addenbrooke's Hospital, Hills Road, Cambridge, CB2 0QQ, UK). The study was approved by the local research ethics committee (REC reference no. 07/Q0106/63) and all patients provided written informed consent. Eligible patients were those women with metastatic breast cancer currently undergoing active treatment. Prior adjuvant therapy and prior treatment of metastatic disease were permitted. All patients had Eastern Cooperative Oncology Group (ECOG) scores of performance status ranging from 0 to 2. Estrogen receptor status and HER2 status were determined according to local guidelines. Women were recruited to the study between April 2010 and April 2012.

Serial blood samples were collected from all participants throughout the study at intervals of ≥ 3 weeks. A total of 30ml of blood was collected at each visit (20 ml in EDTA tubes (Starstedt) and 10 ml in a CellSave[®] tube (Veridex)). Clinical details and results of all imaging performed during the study were collected. For participants with measurable disease, a blinded review of all computed tomography (CT) imaging was conducted to document objective response or progressive disease according to RECIST criteria (version 1.1).¹

Sample processing

Isolation and enumeration of circulating tumor cells

Blood collected in CellSave[®] tubes was processed within 96 hours of sample collection, for the enumeration of circulating tumor cells (CTCs) using the CellSearch[®] system (Veridex) according to the manufacturer's instructions. In brief, this is a semi-automated processing system which isolates CTCs based on immunomagnetic separation using EpCAM (epithelial cell adhesion

molecule) antibody-coated magnetic beads followed by staining with antibodies to DAPI (nuclear dye 4', 6-diamidino-2-phenylindole), cytokeratins (8, 18 and 19) and CD45 (common leukocyte antigen). Images of the cells are then displayed and CTCs can be counted manually. The criteria used to define a cell as a CTC include round to oval morphology, a visible nucleus (DAPI positive), positive staining for cytokeratins and negative staining for CD45. A CellSearch[®] control sample was run in parallel to each blood sample used for CTC enumeration. Enumeration of CTCs was performed blinded to the results of imaging or circulating nucleic acid assessment.

Processing of plasma and extraction of circulating DNA

Blood collected in EDTA tubes was processed within one hour of sample collection and centrifuged at 820 g for 10 min to separate the plasma from the peripheral blood cells. The plasma was then further centrifuged at 20,000 g for 10 min to pellet any remaining cells. The plasma was then stored at -80°C until DNA extraction. DNA was extracted from 2 ml aliquots of plasma using the QIAamp circulating nucleic acid kit (Qiagen) according to the manufacturer's instructions. The DNA was eluted into 50 µl buffer AVE and stored at -20°C. As an internal control to check the yield of DNA extraction, we spiked into each sample equal amounts of a single DNA amplicon, PCR-amplified from *Xenopus tropicalis* genomic DNA. We quantified the amount of spike-in DNA in each eluted DNA sample by digital PCR, and estimated the yield of extraction by comparing the quantity to a no-extraction control that was prepared by re-suspension of the same amount of *Xenopus* DNA in the same elution medium.

Measurement of CA 15-3

Aliquots of 50 µl of plasma were used to measure the levels of CA 15-3 using the ADVIA Centaur[®] immunoassay system (Siemens Healthcare) according to manufacturer's instructions. In brief, the assay is a two-step sandwich immunoassay using direct, chemiluminescent technology. It involves the use of two monoclonal mouse antibodies, DF3 and 115D8, which are specific for CA 15-3. The assays were performed at the Department of Clinical

Biochemistry and Immunology at Addenbrooke's Hospital, Cambridge, which is accredited by Clinical Pathology Accreditation (UK) Limited. The upper limit of normal for the assay is defined as 32.4 U/ml (99th percentile of 399 apparently healthy women aged over 18 years). The assay is standardized by the manufacturer to their own internal standard as there is currently no external reference standard for this analysis. The within-run coefficient of variation was less than 3.7% across the reported range. Quality assurance material was provided by UKNEQAS (Sheffield UK).

Processing and DNA extraction from primary tumor samples

Archived tumor tissue, derived from the primary breast cancer or from a site of metastatic disease, was obtained for all study participants. For fresh frozen tissue samples, 15 x 30 µm sections were used for DNA extraction. DNA was extracted using the DNeasy tissue kit (Qiagen) according to manufacturer's instructions. For formalin fixed paraffin embedded samples (FFPE), H&E sections were reviewed by a pathologist and areas of tumor with >80% cellularity were selected. A 1.5 mm core was then taken from the area of tumor and used for DNA extraction. DNA was extracted using the Ex-WaxTM DNA extraction kit (Millipore) according to manufacturer's instructions.

In parallel to the processing of tumor tissues, matched normal DNA was obtained from all study participants to enable confirmation of somatic versus germline genetic events. This was derived from normal peripheral blood leucocytes (NPBL). After the collection of plasma from each blood sample, the remaining buffy coat containing NPBLs was removed and subjected to red cell lysis using a red cell lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃ and 0.1 mM EDTA pH 7.4). DNA was then extracted from the NPBLs using a standard phenol-chloroform extraction protocol.

Identification of somatic genomic alterations

Identification of somatic mutations by TAm-Seq

The principles and procedures of tagged-amplicon deep sequencing (TAm-Seq) have been described elsewhere.² Libraries for TAm-Seq were prepared using the 48.48 Access Array™ system (Fluidigm). For each individual, 50 ng of tumor and matched normal DNA was amplified with tagged target-specific primers in a microfluidic device, which allowed 48 samples to be analyzed simultaneously with multiplexed assays in 2,304 reaction chambers. We used a panel of amplicons that included coding regions of *TP53* and selected regions in *PIK3CA*.

For FFPE samples, 50 ng DNA from each case were initially subjected to pre-amplification in a 10-μl reaction volume containing both forward and reverse target-specific primers using the FastStart High Fidelity Enzyme Blend (Roche).² The pre-amplified samples were diluted 5-fold in PCR-grade water prior to amplification on the Access Array™ system. DNA from fresh frozen tissue was not pre-amplified and 50 ng DNA was used directly for amplification on the Access Array™ system. Following amplification, products were harvested, tagged with sample-specific barcodes, pooled together and purified using AMPure XP beads.² All samples were analyzed in duplicate to control for errors arising during the PCR reaction. The purified libraries were then sequenced using 100 bp read length on the GAllx or HiSeq Genome Analyzers (Illumina). Reads were demultiplexed according to the barcodes. Each set of reads was aligned independently to the hg19 reference genome using bwa-short in the single-end mode.³ To identify mutations, the data were analyzed as described previously using a confidence margin of 0.9995 with at least 10 representative reads in each duplicate.²

Identification of somatic mutations and structural variants by pair-end whole genome sequencing

Genomic libraries from tumor and matched normal tissue were prepared for nine individuals using the standard Illumina paired-end sample preparation kit

according to the manufacturer's instructions. For eight of the nine cases, DNA fragments of 500 bp in size were sequenced using paired-end 100 bp reads on a HiSeq2000 instrument (Illumina) achieving an average depth of 10-fold. Sequencing reads were aligned to the human genome (hg19) using BWA and data were analyzed using two recently published algorithms for structural variant detection: SVMerge and CREST.^{4,5} Candidate structural rearrangements were then validated and confirmed as somatic by Sanger sequencing of tumor and germline DNA, enabling the putative breakpoint to be identified. For the final case, DNA fragments of 300 bp in size were sequenced using paired-end 100 bp reads on a HiSeq2000 instrument (Illumina) achieving a depth of >30-fold. Sequencing reads were aligned to the human genome (hg19) using ELAND (Illumina) and subsequent data analysis was performed using the Casava 1.8 analysis pipeline (Illumina) to identify somatic single nucleotide variations. Candidate mutations were then validated and confirmed as somatic by Sanger sequencing of tumor and germline DNA. All cases which were analyzed by whole genome sequencing, also had copy number analysis performed using the genome wide human SNP array 6.0 (Affymetrix), with analysis performed using a previously published algorithm.⁶

Quantification of circulating tumor-specific DNA

Digital PCR

Digital PCR was performed using the BioMark™ 12.765 Digital Array (Fluidigm) following manufacturer's definitions and protocols. TaqMan®-based quantitative PCR assays were designed to specifically detect point mutations and corresponding wild-type alleles (Table S6). For patient-specific structural variants, we designed primers flanking the breakpoints identified from Sanger sequencing (Table S7). All assays were designed to ensure that amplicon sizes were between 60 bp and 110 bp (Tables S6 and S7), as circulating DNA in plasma is largely fragmented.^{7,8} The specificity of the assays was confirmed by testing in the matched normal peripheral blood leucocyte DNA of each individual. Different assays were tested on germline DNA, and no mutations

were detected in a total of more than 10,000 wild-type copies. This is consistent with a previous report using this platform, where the false positive rates were less than 1 mutant allele per 3,000 wild-type copies.¹³

For point mutations, 3.5 µl of template DNA was mixed with primers and probes, 1x TaqMan® Universal PCR Master Mix (Applied Biosystems) and 1x sample loading buffer. For structural variants, 3.5 µl of template DNA was mixed with 1000 nM forward and reverse primers in the presence of 1x TaqMan® Gene expression Master Mix (Applied Biosystems), 1x EvaGreen® DNA binding dye (Biotium)⁹ and 1X DNA Binding Dye Sample Loading Reagent (Fluidigm). The cycling profile was as follows: initiation at 50°C for 2 min and incubation at 95°C for 10 min followed by at least 55 cycles of 95°C for 15 s and 56°C or 60°C for 1 min.

Each sample was analyzed by at least two replicates (Fig. S3C). The root mean square (RMS) error (coefficient of variation, CV) was 0.17 for samples with more than 25 estimated targets per assay (see below), and 0.28 overall (due to high sampling noise at low target numbers, with expected CV of $1/(\sqrt{N})$, i.e. >0.2 for less than 25 estimated targets). Quantification by assays based on TaqMan® chemistry or DNA-binding dye (EvaGreen®) for the same mutation had an RMS error of 0.11 (Fig. S3D and Fig. S3E). For extraction duplicates, RMS error was 0.21 (for estimated targets >25 per panel). The total amount of cell-free DNA was quantified using an assay that targets a non-amplified region in the genome, the *RPP30* gene on chromosome 10.¹⁰

The principles of digital PCR and the use of the BioMark™ system for quantification have been described elsewhere.¹¹⁻¹³ Samples were diluted to ensure that the average concentration of template molecules was less than one per amplification reaction (6 nl chamber), as described previously.¹⁴ The data were analyzed with the Digital PCR Analysis Software v3.02 (Fluidigm). Amplifiable DNA fragments randomly segregate into the 765 reaction chambers¹⁵, and a Poisson correction was applied to determine the number of

positive amplifications to estimate the number of target molecules.¹¹ The platform was linear over several orders of magnitude (Fig. S2A), and sensitive at least down to a fractional mutation concentration in the range of 0.1% (Fig. S2B), limited by sampling noise for very rare alleles.^{13,15}

TAm-Seq

The principle and procedures of using TAm-Seq to quantify mutations in plasma has been described elsewhere.² From each sample, 5 µl of plasma DNA was subjected to library preparation, each in duplicate. Harvested PCR products were tagged with sample-specific barcodes and pooled together before purification. The purified libraries were then sequenced using paired-end 100 bp read length on a HiSeq2500 sequencer (Illumina). Reads were demultiplexed and aligned as previously described.² To detect mutations while maintaining a low false-positive rate, the data were analyzed as described previously, using a confidence margin of 0.95 with at least 10 representative reads in each duplicate.² Mutant fractions were converted into copy numbers as described¹⁶ multiplying by total DNA levels, estimated using the *RPP30* assay described above.

Statistical Analysis

Sensitivity analysis

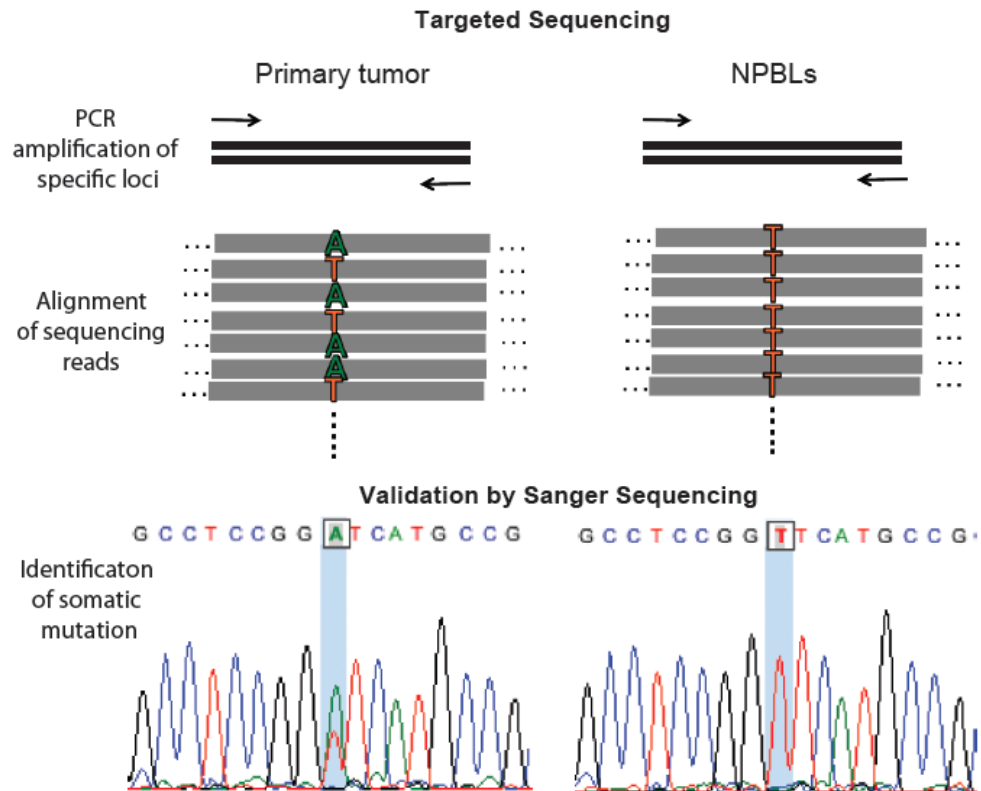
To derive a robust estimate of the sensitivity of each of the circulating biomarkers, a modified bootstrapping method was used.¹⁷ The complete dataset was randomly sampled to obtain a new dataset that contained only one time-point from each individual. This random sampling was then repeated 1000 times, to obtain 1000 datasets each containing independent observations. For each of these datasets, the sensitivity of each biomarker was calculated. The median sensitivity and median difference in sensitivity of the biomarkers was then calculated across the 1000 datasets. This analysis was performed separately for ctDNA and CA 15-3, and then for ctDNA and CTCs. The percentile method was used to obtain 95% confidence intervals.

Survival Analysis

The survival analysis was performed by fitting a different Cox regression model for each variable of interest (ctDNA, CTC and CA15-3). Each model was formulated using the counting process notation (start, end, event)¹⁸ and the predictors were modeled as time dependent covariates using splines to account for non-linear relationships. For each 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. Estimated survival curves were computed for different values of the covariates (quantiles 25%, 50%, 75% and 95%) at the time of 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. The proportional hazards assumption was checked using the `cox.zph` function in the R package `survival`.¹⁹ The models were fitted using R `rms` package.²⁰

Figure S1

a



b

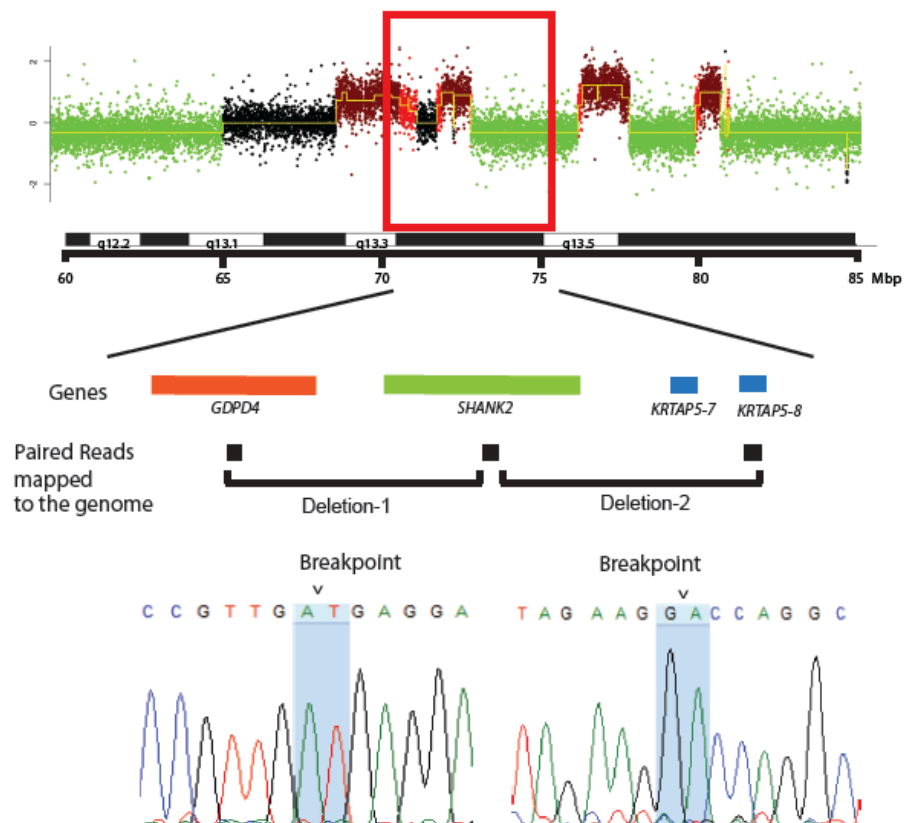


Figure S1

c

Case	Sequencing strategy	Somatic mutations	Somatic SVs	Case	Sequencing strategy	Somatic mutations	Somatic SVs
1	TS	<i>PIK3CA</i> (exon21)		16	TS	<i>PIK3CA</i> (exon21) <i>TP53</i> (exon8)***	
2	TS	<i>TP53</i> (exon7)		17	TS, WGS	-	Deletion (chr 8)
3	TS, WGS	<i>PIK3CA</i> (exon10) <i>PIK3CA</i> (exon21)	Translocation t(6:8) Inversion (chr 8)	18	TS	<i>PIK3CA</i> (exon10) <i>TP53</i> (exon7)	
4	TS, WGS	Multiple point mutations**		19	TS, WGS	-	Deletion-1 (chr 17) Deletion-2 (chr 20)
5	TS	<i>PIK3CA</i> (exon10)		20	TS	<i>TP53</i> (exon6)	
6	TS, WGS	<i>PIK3CA</i> (exon10)	Deletion-1* (chr 11) Deletion-2* (chr 11) Deletion-3* (chr 11)	21	TS	<i>TP53</i> (exon5)	
7	TS, WGS	<i>PIK3CA</i> (exon21)	Deletion (chr 11)	22	TS	<i>TP53</i> (exon5)	
8	TS	<i>TP53</i> (exon8)		23	TS	<i>TP53</i> (exon8) <i>PIK3CA</i> (exon21)***	
9	TS	<i>PIK3CA</i> (exon10)		24	TS	<i>TP53</i> (exon4)	
10	TS	<i>TP53</i> (exon7)		25	TS	<i>TP53</i> (exon5)	
11	TS, WGS	-	Translocation t(6:8)	26	TS	<i>TP53</i> (exon5) <i>PIK3CA</i> (exon21)	
12	TS, WGS	-	Deletion (chr 8)	27	TS	<i>TP53</i> (exon5)	
13	TS, WGS	-	Translocation t(1:17)	28	TS	<i>TP53</i> (exon7)	
14	TS	<i>PIK3CA</i> (exon10)		29	TS	<i>TP53</i> (exon8)	
15	TS	<i>TP53</i> (exon7)		30	TS	<i>TP53</i> (exon5)	

Figure S1

Identification of somatic genomic alterations

a. Somatic mutations were identified by targeted sequencing and validated by Sanger sequencing. A representative example of a *TP53* mutation identified for case 18 is shown. NPBL=normal peripheral blood leucocytes.

b. Structural variants were identified from paired-end whole genome sequencing data and validated by Sanger sequencing. Representative examples of 2 structural variants identified for case 6 are shown. These structural variants occurred in the setting of a complex rearrangement associated with a region of amplification. Copy number analysis was performed using the genome wide human SNP 6.0 array (Affymetrix) (see Supplementary Methods).

c. Somatic genomic alterations used as biomarkers in individual cases. TS=targeted sequencing (using TAm-Seq), WGS=whole genome sequencing, SV=structural variants.

* Deletions occurring in the setting of a complex rearrangement associated with amplification.

** Multiple somatic point mutations in *PIK3CA*, *ZFYVE21*, *CD1A*, *IQCA1*, *MET* and *KIAA0406* (see Table S2).

*** Mutations identified in plasma that were not detected in the tumor.

Figure S2

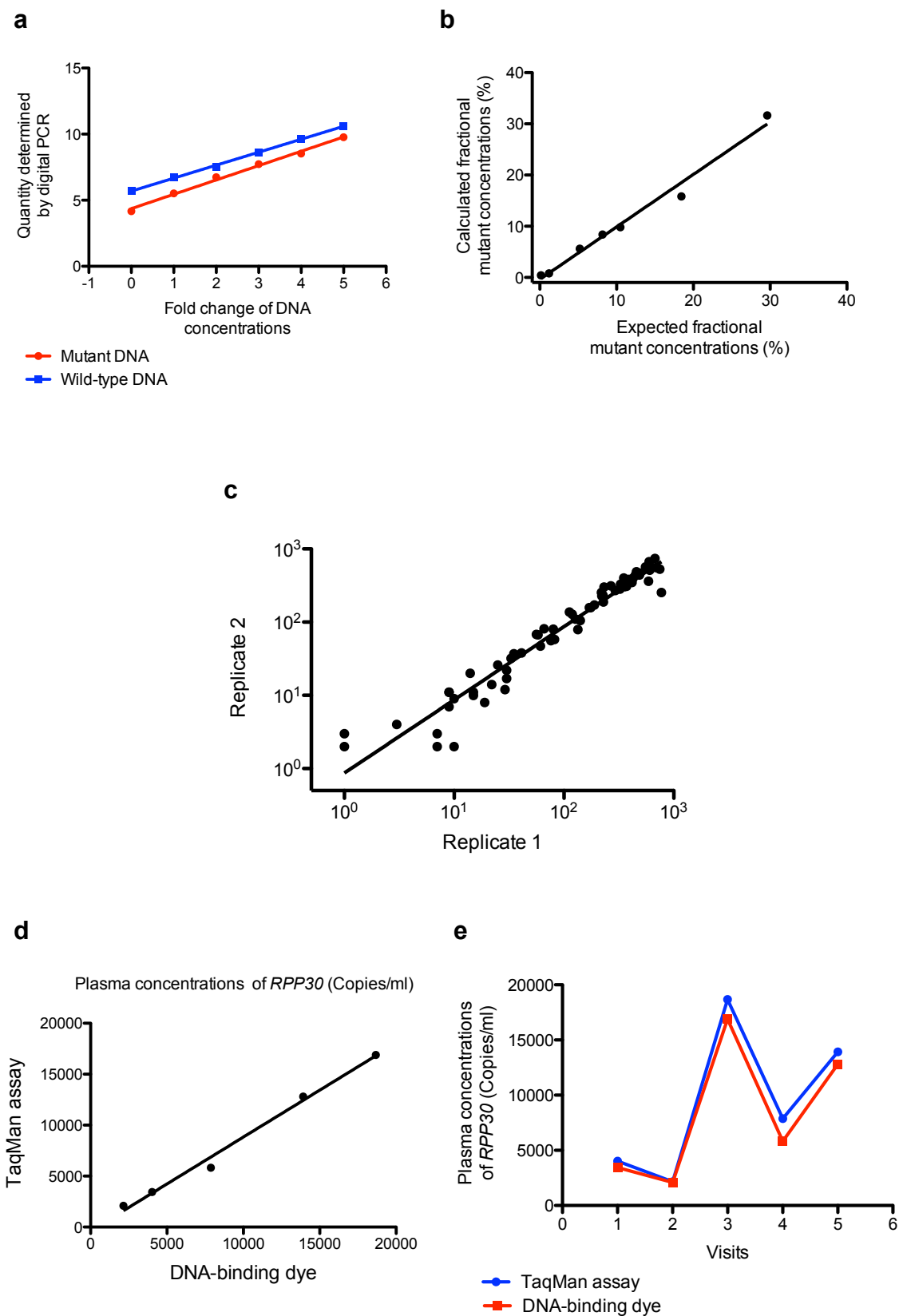


Figure S2

Quantitative performance of digital PCR.

a. A serial 2-fold dilution of tumor DNA carrying a *PIK3CA* mutation was analyzed by digital PCR. Quantification by digital PCR (log2 of estimated targets) was linear over >2 orders of magnitude of dilution.

b. A plasma sample carrying *PIK3CA* mutant sequences was mixed with wild-type plasma DNA such that the fractional mutation concentrations ranged from 0.1% to 30%. The mixtures were analyzed with digital PCR. Mutant molecules were detected at concentrations as low as 0.1% expected allele fraction. Root mean square error (coefficient of variation) between expected and observed mutant allele fraction was 0.06 for allele fractions >2% (>25 estimated mutant targets).

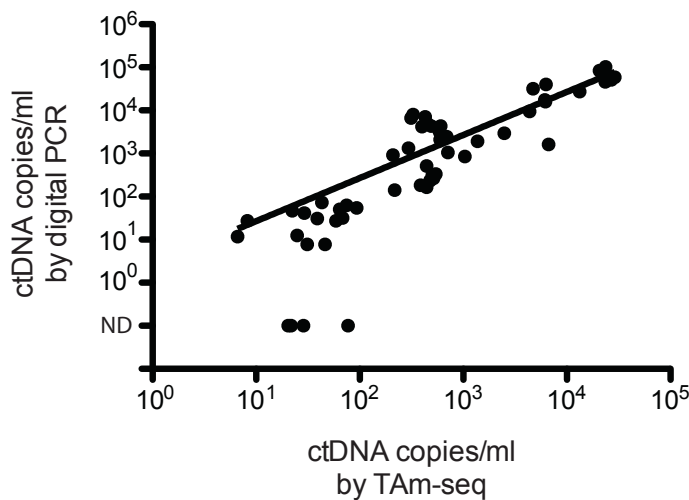
c. Reproducibility of estimated targets determined by digital PCR between replicates. The root mean square (RMS) error (coefficient of variation) was 0.17 for samples with more than 25 estimated targets per assay, and 0.28 overall.

d. Quantification by assays based on TaqMan[®] chemistry or DNA-binding dye for the same mutation had an RMS error of 0.11.

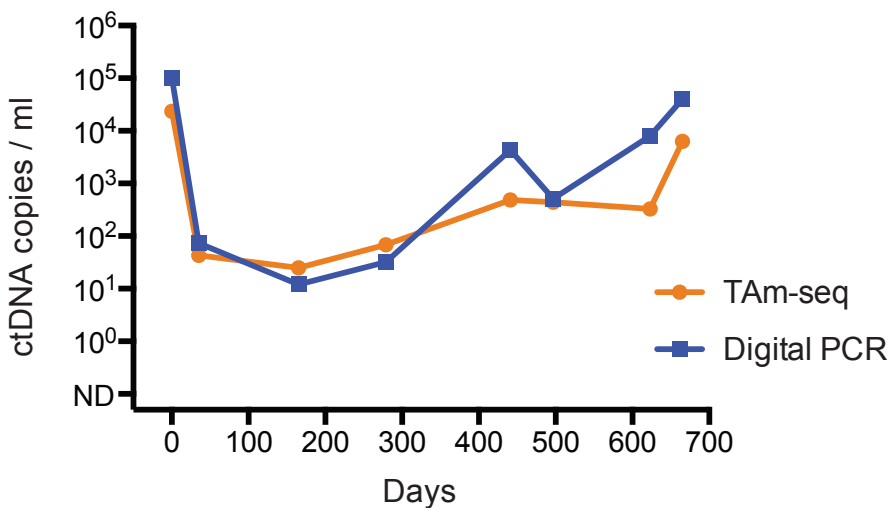
e. Plasma concentrations of *RPP30* DNA at visits 1-5 for case 6 were quantified by two different assays based on TaqMan[®]-chemistry and a DNA-binding dye (Evagreen[®]), respectively.

Figure S3

a



b



c

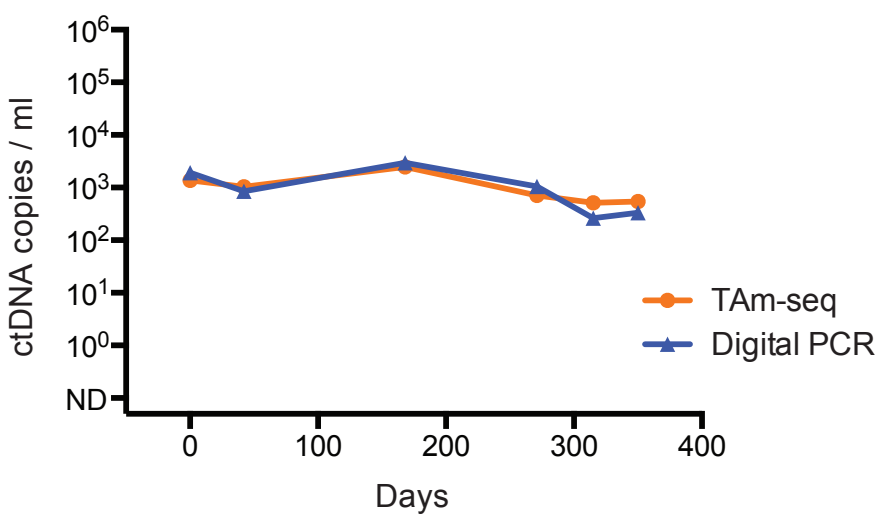


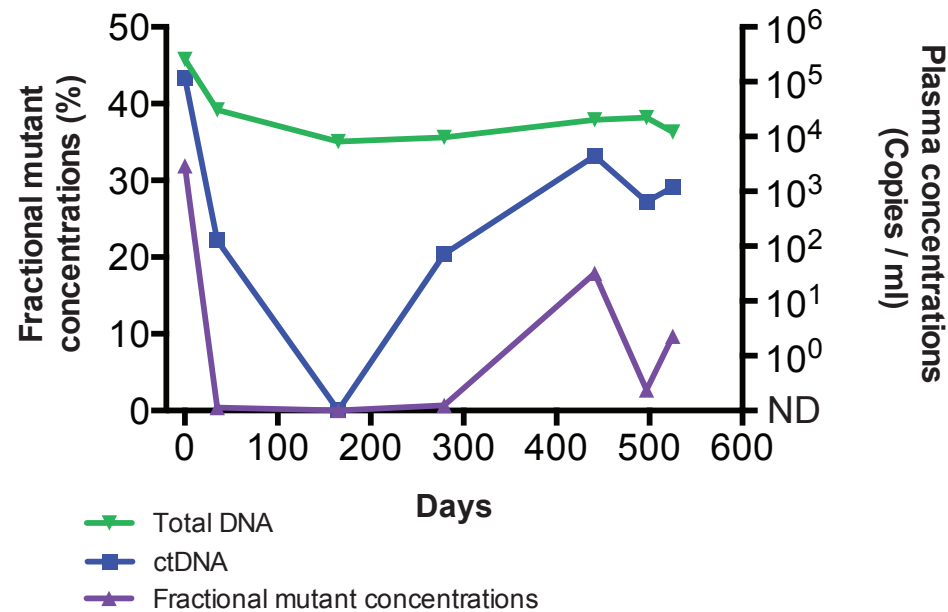
Figure S3

Comparison of the quantitative performance of digital PCR and TAm-Seq

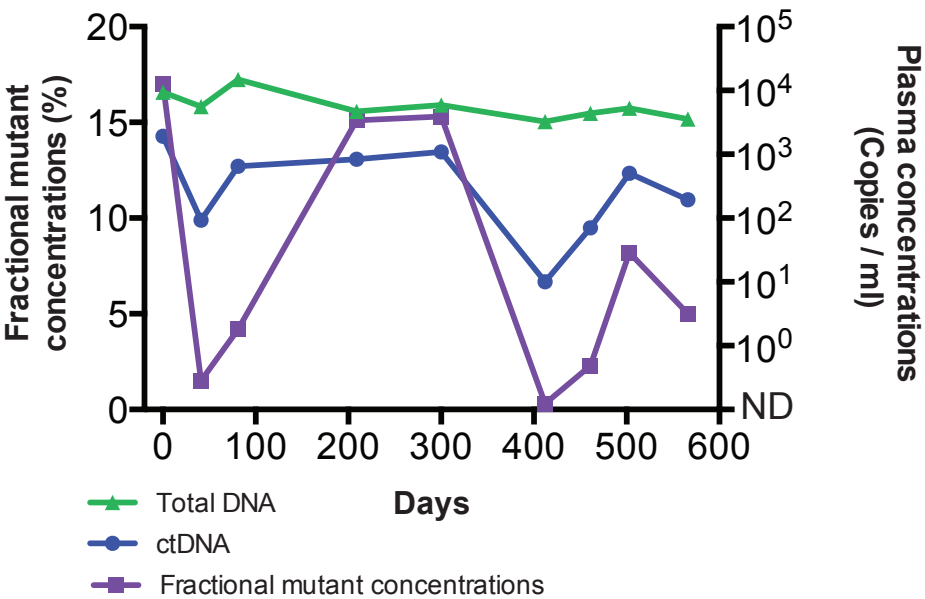
- a. ctDNA levels (copies/ml) of 7 mutations across 14 time points from cases 4 and 23 were obtained separately using digital PCR and TAm-Seq and compared. Levels of ctDNA calculated by the two methods were significantly correlated with each other (Spearman Correlation, $r=0.878$, $p\text{-value}<0.0001$).
- b. Plasma levels of tumor specific *PIK3CA* (copies/ml) as calculated by digital PCR and TAm-Seq for case 4. ND denotes not detected.
- c. Plasma levels of tumor specific *PIK3CA* (copies/ml) as calculated by digital PCR and TAm-Seq for case 23. ND denotes not detected.

Figure S4

a



b



Comparison of plasma concentrations of tumor specific and total DNA

Plasma concentrations of total DNA (copies/ml), tumor-specific *PIK3CA* DNA (copies/ml) and fractional concentrations (%) of mutant *PIK3CA* for (a) Case 4 and (b) Case 5. Total DNA in both cases showed limited dynamic range compared to ctDNA carrying the *PIK3CA* mutation. ND denotes not detected.

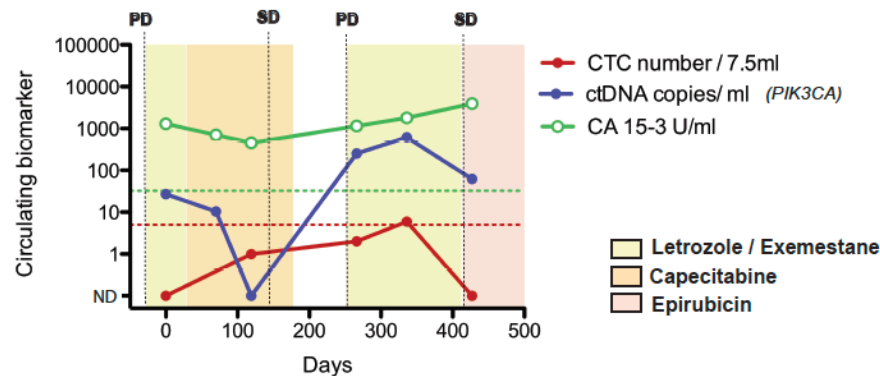
Figure S5 (see following pages)

Serial ctDNA levels, CA 15-3 levels, CTC numbers and imaging of individual cases

Serial measurements of ctDNA (copies/ml plasma), CA 15-3 (U/ml), CTCs (per 7.5 ml whole blood) and medical imaging during serial follow up of 20 individual cases (a-t). Details of therapy (endocrine, cytotoxic or biological therapies) are indicated by colored shading. Red dotted line indicates 5 CTCs per 7.5 ml blood. Green dotted line indicates CA 15-3 of 32.4U/ml. ND=not detected. All imaging was performed using computed tomography (CT), and assessed according to RECIST criteria: SD=stable disease, PR=partial response, PD=progressive disease, NM=no measurable disease.

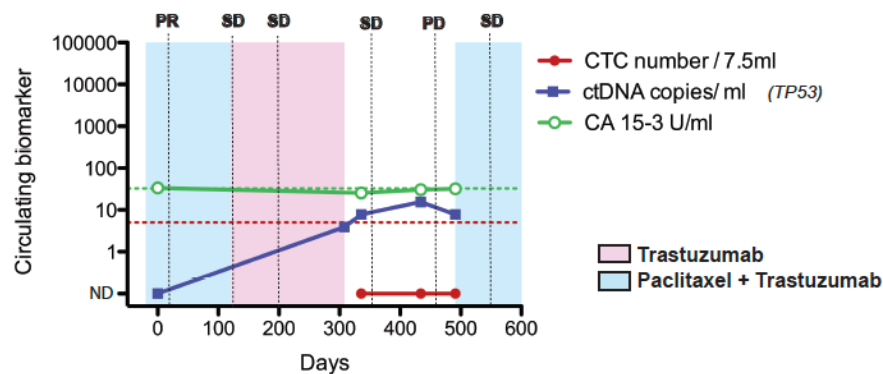
Figure S5

a Case 1



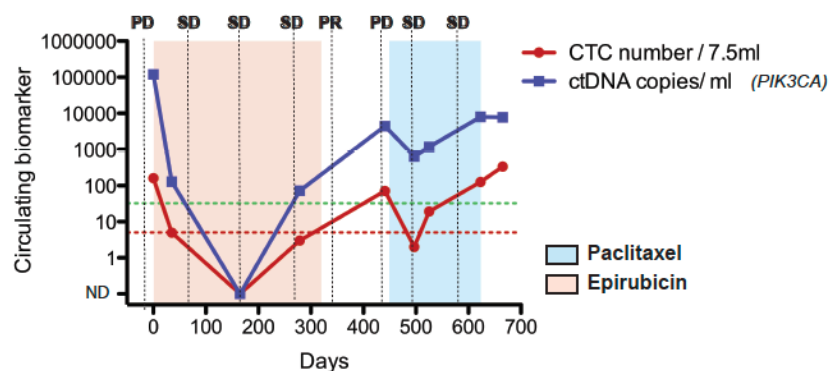
66 year old with ER positive, HER2 negative breast cancer with bone and visceral metastases (lung). She had documented progressive disease whilst receiving letrozole and was switched to capecitabine. Her disease stabilised following commencement of capecitabine and she subsequently had a break from treatment. Exemestane was started upon further disease progression. This was changed to epirubicin because of symptomatic deterioration despite the appearance of stable disease by RECIST criteria.

b Case 2



60 year old with ER positive, HER2 positive breast cancer with visceral metastases (lung). She had stable disease following treatment with paclitaxel and trastuzumab. Trastuzumab was continued post cessation of paclitaxel but was later stopped due to toxicity. Disease progression was observed whilst she was on a break from treatment and paclitaxel was subsequently recommenced.

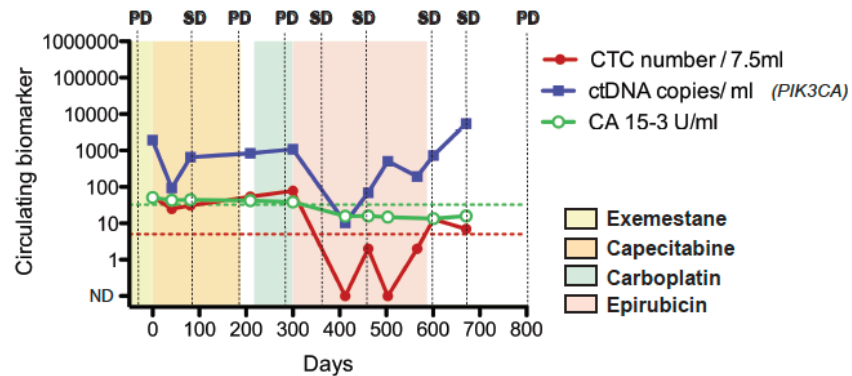
c Case 4



70 year old with ER positive, HER2 negative breast cancer with bone and visceral metastases (liver). She had documented progressive disease prior to the commencement of epirubicin with subsequent successful disease stabilisation. Further disease progression was observed after the cessation of treatment and paclitaxel was commenced. This was later ceased due to toxicity. She subsequently died on day 732 of follow up.

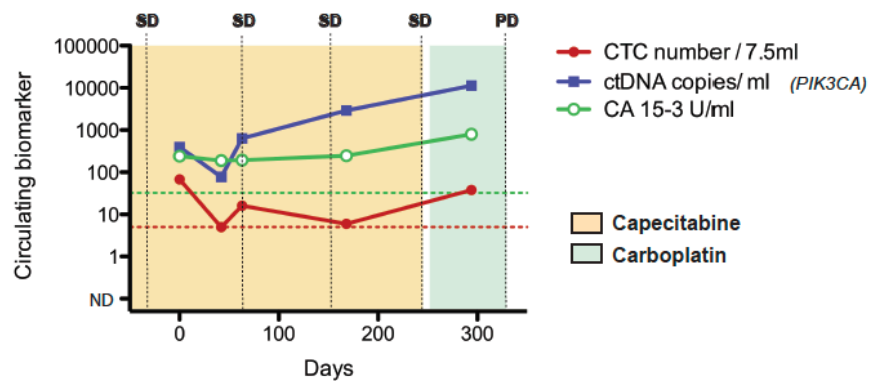
Figure S5

d Case 5



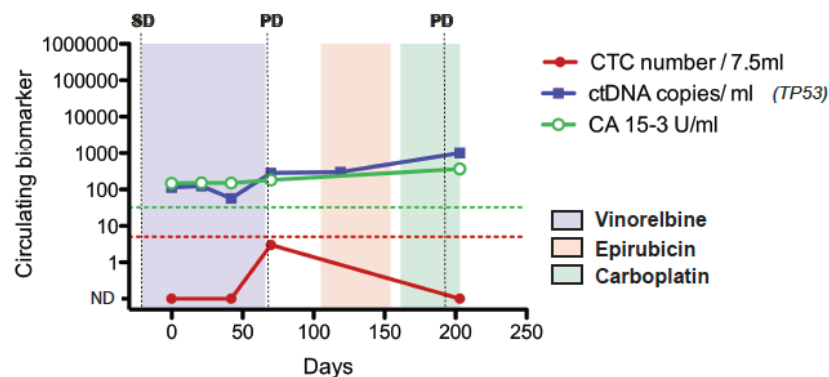
60 year old with ER positive, HER2 negative breast cancer with bone and visceral metastases (liver). Disease progression was observed whilst on exemestane, then following capecitabine and carboplatin. Disease stabilisation was achieved after commencement of epirubicin. The epirubicin was subsequently ceased after she received her maximum accumulative dose of anthracycline.

e Case 6



76 year old with ER positive, HER2 negative breast cancer with bone and visceral metastases (lung). Repeated imaging showed stable disease by RECIST throughout capecitabine therapy, however an increasing pleural effusion led to a change in therapy to carboplatin. Progressive disease was documented on CT following the commencement of carboplatin and she died shortly after.

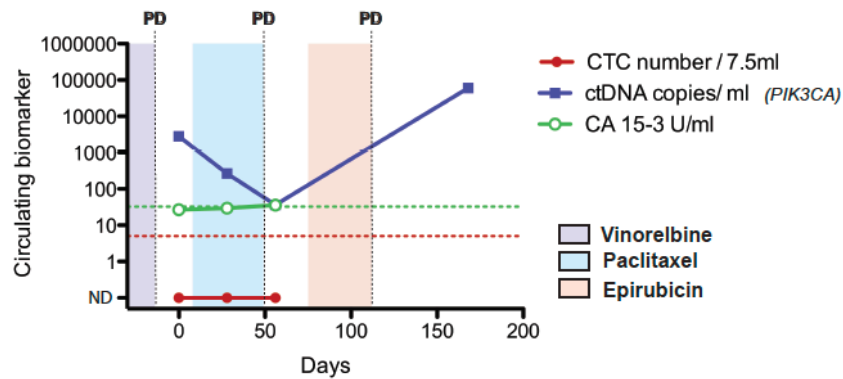
f Case 8



56 year old with ER positive, HER2 positive breast cancer with bone and visceral metastases (liver). Progressive disease developed on vinorelbine. Epirubicin was subsequently commenced but stopped due to toxicity. Carboplatin was then commenced but progressive disease was again established following treatment. Trastuzumab had been previously discontinued due to toxicity. She subsequently died at day 366 of follow up.

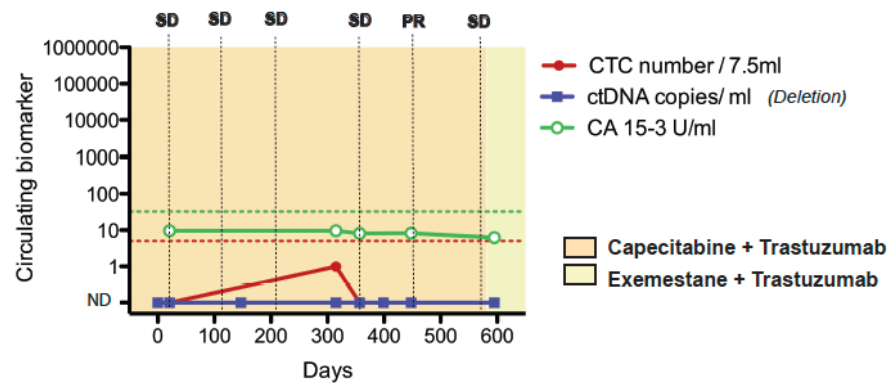
Figure S5

g Case 9



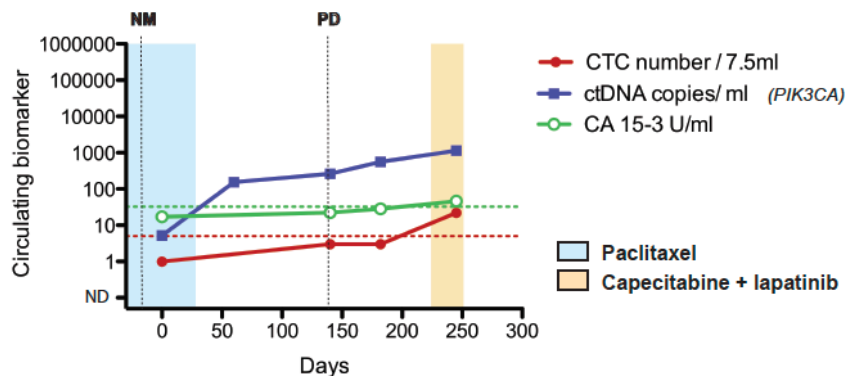
75 year old with ER negative, HER2 negative breast cancer with bone and visceral metastases (lung, liver). Disease progression was documented by RECIST criteria after commencement of paclitaxel, however, the CT scan did show evidence of a mixed response. Epirubicin was subsequently commenced with further disease progression. She died on day 204 of follow up.

h Case 12



68 year old with ER positive, HER2 positive breast cancer with visceral metastases. Her systemic disease consisted of small volume mediastinal lymphadenopathy which remained stable during treatment with capecitabine. Her treatment was eventually changed to exemestane.

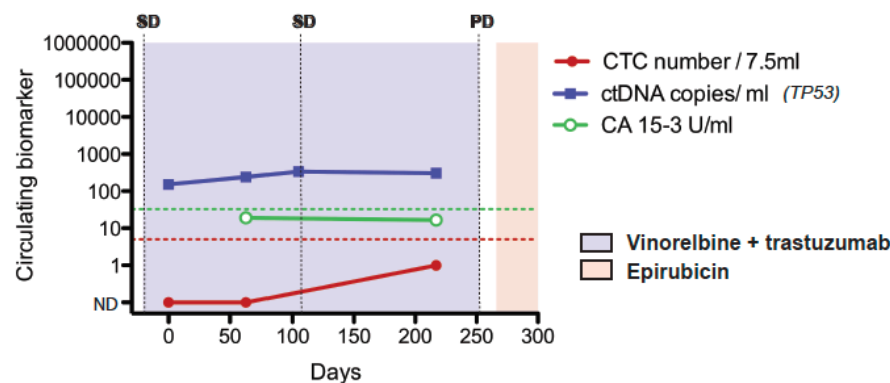
i Case 14



59 year old with ER positive, HER2 positive breast cancer with visceral metastases. Her original metastatic disease involved extensive skin infiltration which was not measurable by RECIST criteria on CT. Progressive disease subsequently developed with axillary lymphadenopathy and lymphangitis carcinomatosa prior to the commencement of capecitabine and lapatinib. She died on day 301 of follow up.

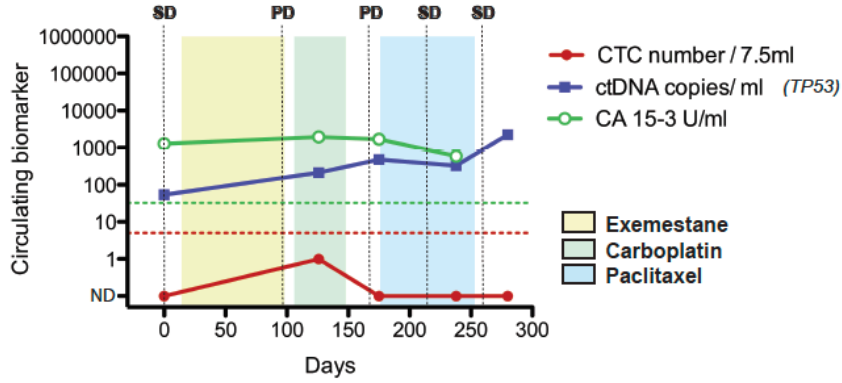
Figure S5

j Case 15



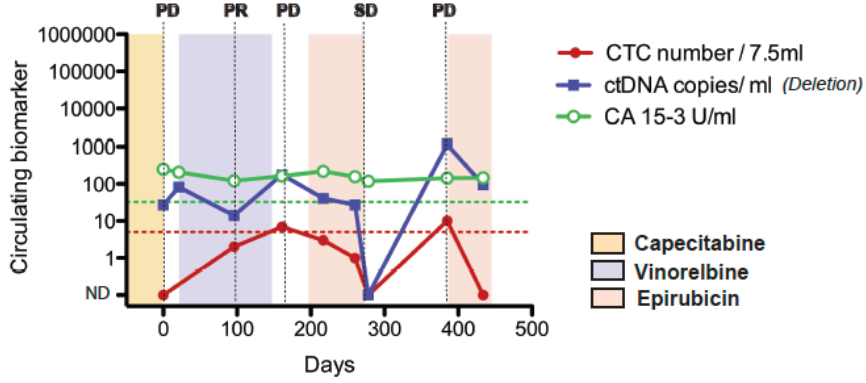
54 year old with ER positive, HER2 positive breast cancer with bone and visceral metastases (lung, liver, cerebral). She developed progressive pulmonary and liver disease whilst receiving vinorelbine and trastuzumab and her therapy was subsequently changed to epirubicin. She died on day 389 of follow up.

k Case 16



70 year old with ER positive, HER2 negative breast cancer with bone and visceral metastases (lung, liver). She developed progressive disease following exemestane, and then following carboplatin. Paclitaxel chemotherapy was then initiated with subsequent disease stabilisation. She later died on day 332 of follow up.

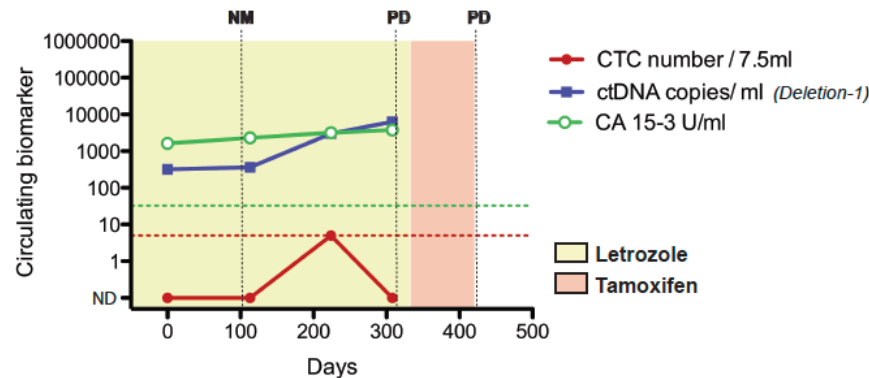
l Case 17



47 year old with ER positive, HER2 negative breast cancer with bone and visceral metastases (lung, liver, cerebral). She developed progressive disease following capecitabine and was started on vinorelbine with initially a good partial response. She subsequently developed progressive disease with increasing liver and new cerebral metastases and epirubicin was initiated in conjunction with whole brain radiotherapy. Following a break from treatment, further disease progression was identified in the liver and she was re-commenced on epirubicin.

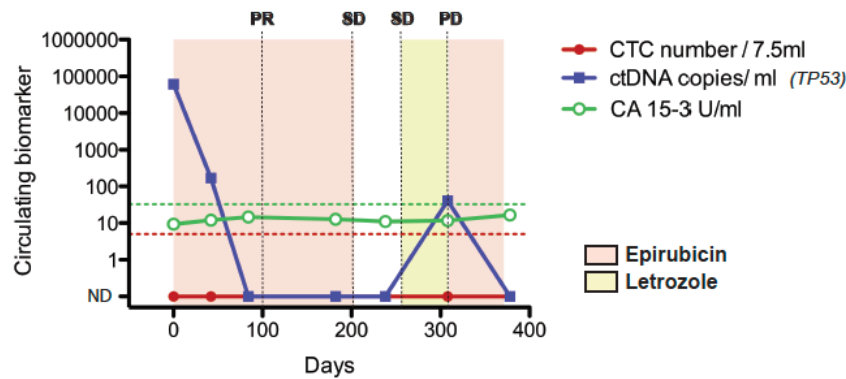
Figure S5

m Case 19



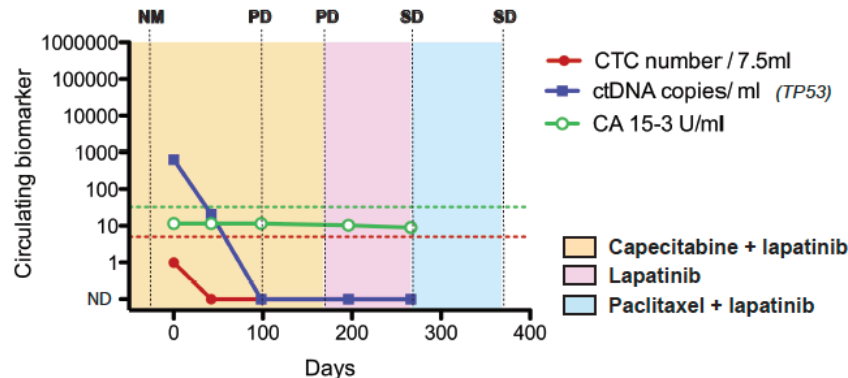
83 year old with ER positive, HER2 negative breast cancer with bone metastases which were not measurable on CT by RECIST criteria. Following the development of increasing soft tissue nodules in the left breast and a left sided pleural effusion she was changed to tamoxifen. Following commencement of tamoxifen, further imaging showed progressive disease with liver metastases.

n Case 20



73 year old with ER positive, HER2 negative breast cancer with bone and visceral metastases (liver). She had a partial response on epirubicin and was changed to letrozole. Progressive disease was documented following the commencement of letrozole, and epirubicin was re-started.

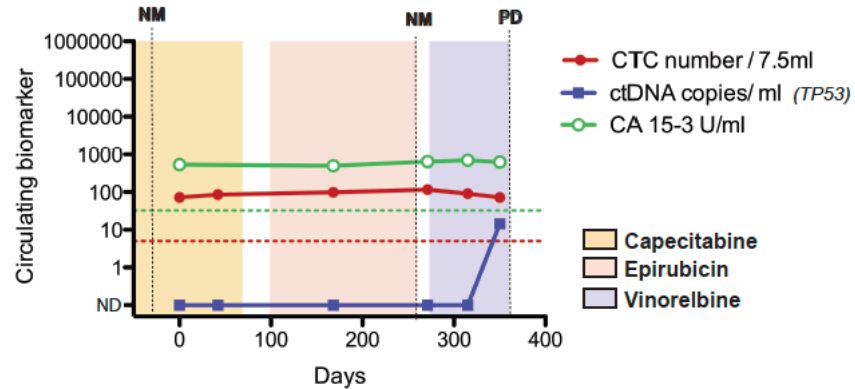
o Case 22



58 year old with ER positive, HER2 positive breast cancer with bone metastases which were not measurable on CT by RECIST criteria. Her progressive disease was marked by the development of cerebral metastases in the absence of progression at other systemic sites. She received whole brain radiotherapy. Further progression was then noted with increasing cerebral metastases, despite stable bone metastases. Her systemic treatment was subsequently changed to paclitaxel and lapatinib.

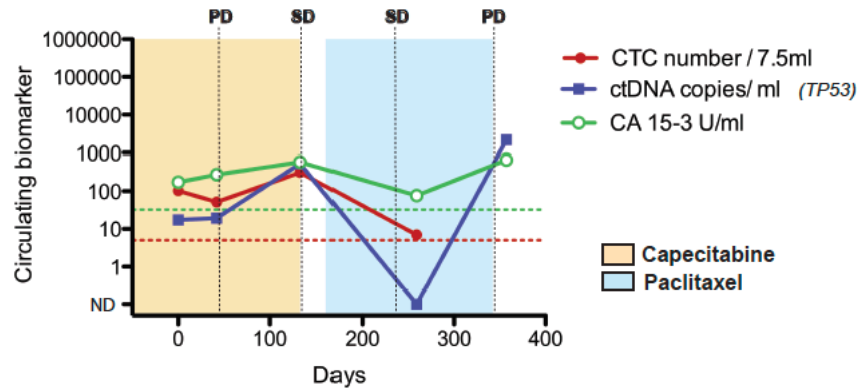
Figure S5

p Case 23



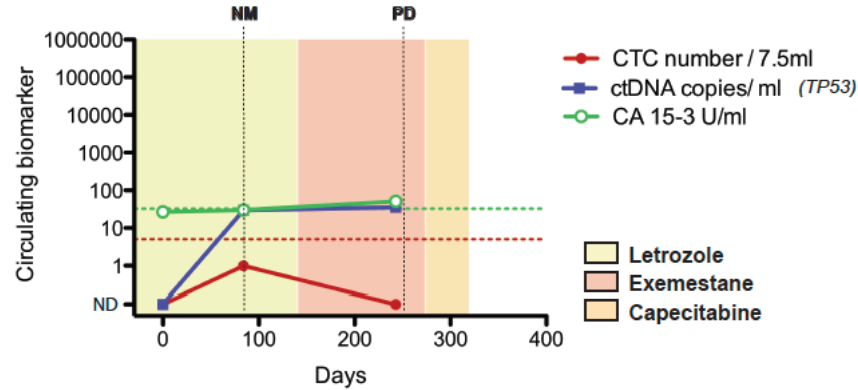
75 year old with ER positive, HER2 negative breast cancer with bone metastases which were not measurable on CT by RECIST criteria. Changes in therapy were initiated based on progressive disease on bone scan. She then developed a new solitary pulmonary metastasis whilst on vinorelbine chemotherapy.

q Case 24



43 year old with ER positive, HER2 negative breast cancer with bone and visceral metastases (lung, liver). She was initially treated with capecitabine and this was eventually ceased due to continued enlargement of pulmonary nodules (although this did not reach criteria for progressive disease by RECIST). Following this she was commenced on paclitaxel until further disease progression was established on CT with the development of liver metastases.

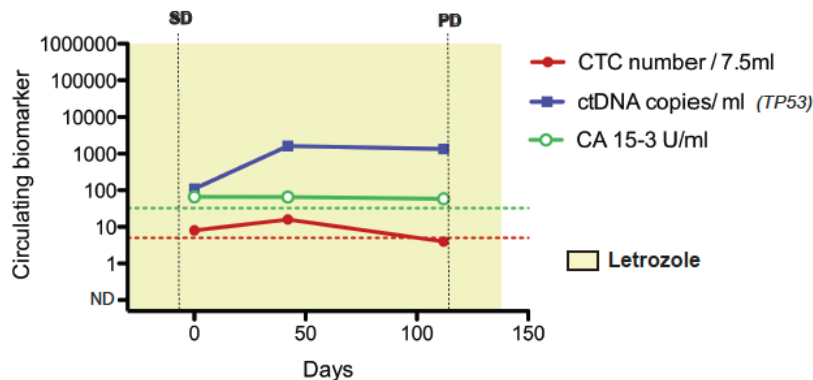
r Case 26



54 year old with ER positive, HER2 negative breast cancer with bone metastases which were initially not measurable on CT by RECIST criteria. New bone metastases were then evident on CT consistent with progressive disease at which time her treatment was changed to capecitabine.

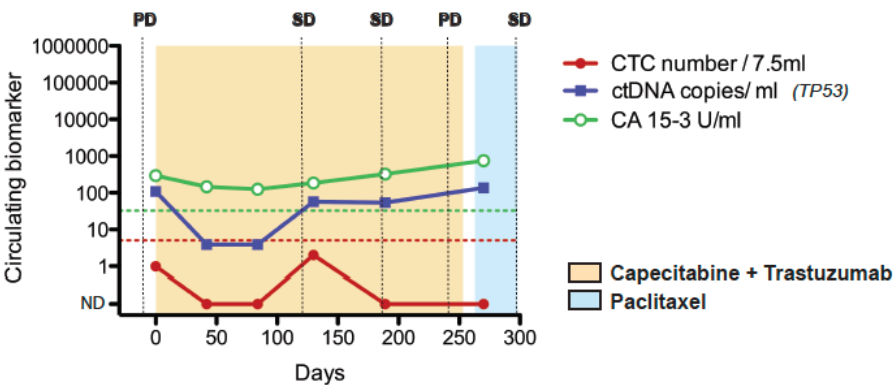
Figure S5

s Case 27



85 year old with ER positive, HER2 negative breast cancer with bone and visceral metastases (lung, liver). She had documented progressive disease whilst on letrozole with the development of liver metastases and died shortly after.

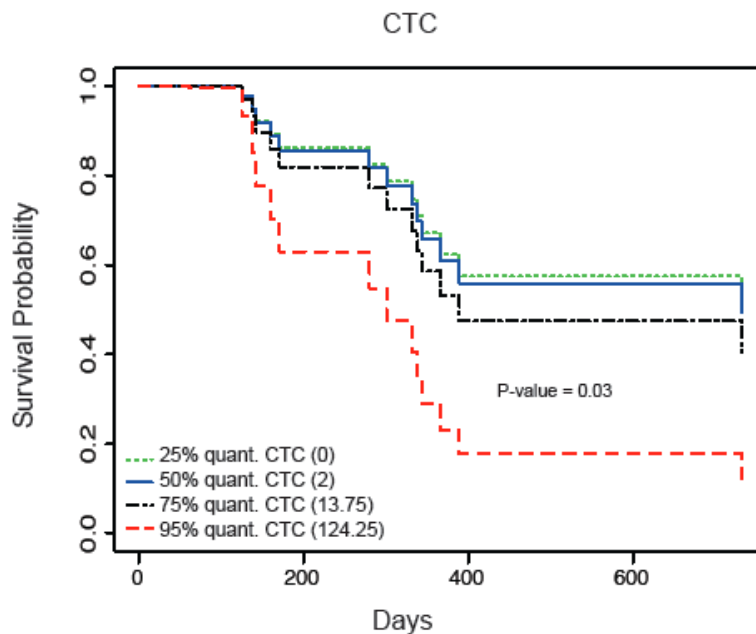
t Case 28



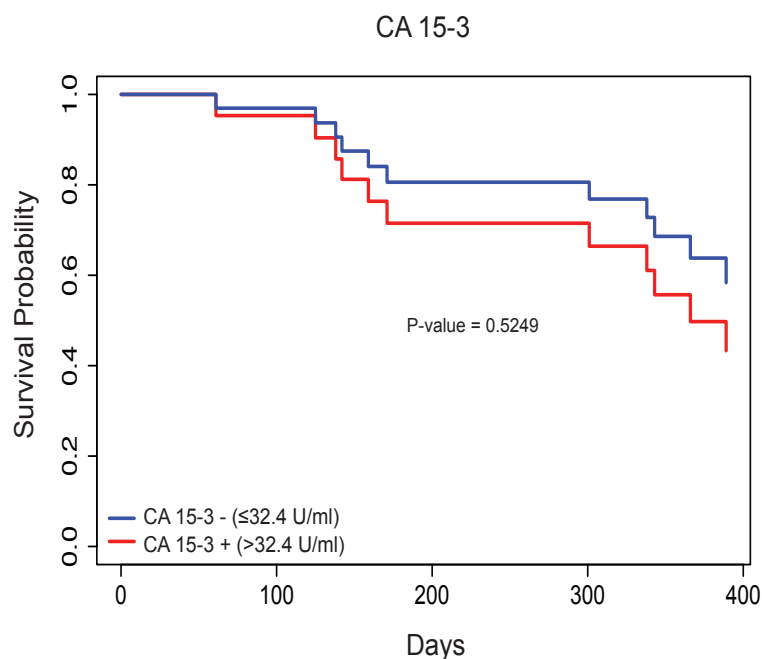
79 year old with ER positive, HER2 positive breast cancer with bone and visceral metastases (lung, liver). She had documented progressive disease with increasing pulmonary and liver metastases whilst receiving capecitabine and trastuzumab. She was subsequently switched to paclitaxel with stabilisation of her disease on CT.

Figure S6

a



b



Prognostic significance of the circulating biomarkers

a. Relationship between CTCs (per 7.5 ml of blood) and overall survival. Increasing CTCs were associated with inferior overall survival (p-value=0.03). The number of individuals at risk at 200, 400 and 600 days were 23, 8 and 3 respectively.

b. Relationship between CA 15-3 (U/ml) and overall survival (p-value=0.52).

Table S1: Clinical characteristics of cases

Case no.	Age (years)	Grade	ER status	HER2 status	Sites of metastatic disease	Follow up on study (days)	Vital Status
1	66	2	+	-	Visceral, non-visceral	501	Alive
2	60	NA	+	+	Visceral	536	Alive
3	56	2	+	-	Visceral, non-visceral	280	Deceased
4	70	2	+	-	Visceral, non-visceral	732	Deceased
5	60	2	+	-	Visceral, non-visceral	717	Alive
6	76	3	+	-	Visceral, non-visceral	338	Deceased
7	63	2	+	+	Non-visceral	651	Alive
8	56	3	+	+	Visceral, non-visceral	366	Deceased
9	75	3	-	-	Visceral, non-visceral	204	Deceased
10	69	3	+	-	Visceral	159	Deceased
11	59	3	+	-	Visceral, non-visceral	171	Deceased
12	68	3	+	+	Visceral	599	Alive
13	45	3	-	-	Visceral, non-visceral	61	Deceased
14	59	3	+	+	Visceral	301	Deceased
15	54	3	+	+	Visceral, non-visceral	389	Deceased
16	70	1	+	-	Visceral, non-visceral	332	Deceased
17	47	2	+	-	Visceral, non-visceral	445	Alive
18	66	3	+	+	Non-visceral	343	Deceased
19	83	3	+	-	Visceral, non-visceral	420	Alive
20	73	3	+	-	Visceral, non-visceral	396	Alive
21	64	3	-	+	Non-visceral, visceral	142	Deceased
22	58	2	+	+	Visceral, non-visceral	368	Alive
23	75	NA	+	-	Visceral, non-visceral	361	Alive
24	43	3	+	-	Visceral, non-visceral	361	Alive
25	61	2	+	-	Visceral, non-visceral	259	Alive
26	54	3	+	-	Non-visceral	319	Alive
27	85	3	+	-	Visceral, non-visceral	138	Deceased
28	79	3	+	+	Visceral, non-visceral	298	Alive
29	85	2	+	-	Visceral, non-visceral	151	Alive
30	64	3	+	-	Visceral, non-visceral	125	Deceased

Table S2: Somatic mutations identified by Tam-Seq or whole genome sequencing
a: Somatic mutations identified by Tam-Seq

Case no.	Tumor Tissue	Gene	Exon	Transcript	Chromosome	Genomic Location	Mutation	cDNA change	Protein change	Mutant allele fraction in tumor by TS (%)
1	Primary FFPE	PIK3CA	Exon 21	NM_006218	3	178952085	A>T	c.A3140T	p.H1047L	27.0
2	Primary FFPE	TP53	Exon 7	NM_000546	17	7577538	C>T	c.G743A	p.R248Q	89.0
3	Primary FF	PIK3CA	Exon 10	NM_006218	3	178952085	G>A	c.G1633A	p.E545K	13.0
3	Primary FF	PIK3CA	Exon 21	NM_006218	3	178952085	A>G	c.A3140G	p.H1047R	23.7
4	Primary FF	PIK3CA	Exon 10	NM_006218	3	178952085	G>A	c.G1633A	p.E545K	20.4
5	Primary FF	PIK3CA	Exon 10	NM_006218	3	178952085	G>A	c.G1633A	p.E545K	10.0
6	Primary FF	PIK3CA	Exon 10	NM_006218	3	178952085	G>A	c.G1633A	p.E545K	30.6
7	Primary FF	PIK3CA	Exon 21	NM_006218	3	178952085	A>G	c.A3140G	p.H1047R	4.8
8	Primary FF	TP53	Exon 8	NM_000546	17	7577096	T>C	c.A842G	p.D281G	17.5
9	Primary FF	PIK3CA	Exon 10	NM_006218	3	178952085	G>A	c.G1633A	p.E545K	51.2
10	Primary FF	TP53	Exon 7	NM_000546	17	7577539	G>A	c.C742T	p.R248W	71.0
14	Primary FF	PIK3CA	Exon 10	NM_006218	3	178952085	G>A	c.G1633A	p.E545K	9.5
15	Primary FFPE	TP53	Exon 7	NM_000546	17	7577566	T>C	c.A715G	p.N239D	63.8
16	Primary FFPE	PIK3CA	Exon 21	NM_006218	3	178952085	A>G	c.A3140G	p.H1047R	13.7
16	Primary FFPE	TP53	Exon 8	NM_000546	17	7577022	G>A	c.C916T	p.R306X	Only identified in plasma
18	Primary FF	PIK3CA	Exon 10	NM_006218	3	178952085	G>A	c.G1633A	p.E545K	12.0
18	Primary FF	TP53	Exon 7	NM_000546	17	7577541	T>A	c.A740T	p.N247I	47.9
20	Primary FFPE	TP53	Exon 6	NM_000546	17	7578190	T>C	c.A659G	p.Y220C	56.2
21	Primary FFPE	TP53	Exon 5	NM_000546	17	7578461	C>A	c.G469T	p.V157F	61.2
22	Primary FFPE	TP53	Exon 5	NM_000546	17	7578442	T>C	c.A488G	p.Y163C	16.0
23	Primary FFPE	TP53	Exon 8	NM_000546	17	7577032	C>T	c.G906A	p.G302G	99.2
23	Primary FFPE	PIK3CA	Exon 21	NM_006218	3	178952085	A>G	c.A3140G	p.H1047R	Only identified in plasma
24	Primary FFPE	TP53	Exon 4	NM_000546	17	7579415	C>T	c.G272A	p.W91X	86.3
25	Primary FF	TP53	Exon 5	NM_000546	17	7578406	C>T	c.G524A	p.R175H	12.4
26	Primary FFPE	TP53	Exon 5	NM_000546	17	7578427	T>C	c.A503G	p.H168R	87.1
26	Primary FFPE	PIK3CA	Exon 21	NM_006218	3	178952085	A>G	c.A3140G	p.H1047R	59.3
27	Primary FFPE	TP53	Exon 5	NM_000546	17	7578502	A>C	c.T428G	p.V143G	77.7
28	Primary FF	TP53	Exon 7	NM_000546	17	7577538	C>T	c.G743A	p.R248Q	16.0
29	Primary FFPE	TP53	Exon 8	NM_000546	17	7577099	C>G	c.G839C	p.R280T	41.6
30	Primary FFPE	TP53	Exon 5	NM_000546	17	7578461	C>A	c.G469T	p.V157F	78.5

b: Multiple somatic mutations identified by whole genome sequencing

Case no.	Tumor Tissue	Gene	Exon	Transcript	Chromosome	Genomic Location	Mutation	cDNA change	Protein change	Mutant allele fraction in tumor by TS (%)
4	Primary FF	PIK3CA	Exon 10	NM_006218	3	178952085	G>A	c.G1633A	p.E545K	20.4
4	Primary FF	CD1A	Exon 4	NM_001763	1	158226762	C>T	c.C791T	p.A264V	24.5
4	Primary FF	IQCA1	Exon 3	NM_024726	2	237402439	G>A	c.C28T	p.A143V	15.8
4	Primary FF	MET	Exon 9	NM_000245	7	116398635	C>T	c.C228T	p.P742L	14.3
4	Primary FF	ZFY/E21	Exon 5	NM_001198953	14	104195486	G>A	c.G493A	p.V165M	32.7
4	Primary FF	KIAA0406	Exon 3	NM_014657	20	36641201	C>T	c.G1018A	p.V340I	20.5

*FFPE = formalin fixed paraffin embedded tissue

**FF = fresh frozen tissue

TS = Tam-Seq

Table S3: Somatic structural variants identified by whole genome sequencing

Case no.	Tumor Tissue	Rearrangement Type	First read		Second Read	
			Chromosome	Position	Chromosome	Position
3	Primary FF	Translocation	6	48758904	8	33085736
		Inversion	8	15836118	8	38717849
6	Primary FF	Deletion-1*	11	70903163	11	76954849
		Deletion-2*	11	70903344	11	71241988
		Deletion-3*	11	70903921	11	71242022
7	Primary FF	Deletion	11	70511989	11	72304497
11	Primary FF	Translocation	6	88044875	8	23307288
12	Primary FF	Deletion	8	137118102	8	141098936
13	Primary FF	Translocation	1	93606972	17	72653198
17	Primary FF	Deletion	8	34253557	8	35857918
19	Primary FF	Deletion-1	17	3714653	17	3913422
		Deletion-2	20	9901456	20	10276583

**FF = fresh frozen tissue
*Deletion occurring in setting of a complex rearrangement associated with amplification

Table S4: Serial quantification of ctDNA, CA 15-3 and CTC levels during therapy

Case no.	Visit no.	CTC no. (per 7.5ml)	CA 15-3 (units/ml)	ctDNA analysis method	Volume of plasma analyzed (µl)	ctDNA (Copies/ml)	Mutant allele fraction (%)	Mutation (1)	Volume of plasma analyzed (µl)	ctDNA (Copies/ml)	Mutant allele fraction (%)	Mutation (2)	Volume of plasma analyzed (µl)	ctDNA (Copies/ml)	Mutant allele fraction (%)	Mutation (3)
1	1	0	1293.5	dPCR	560	27.17	1.59	PIK3CA (Exon 21)								
1	2	NA	702.6	dPCR	420	10.35	0.13	PIK3CA (Exon 21)								
1	3	1	453.9	dPCR	560	ND	ND	PIK3CA (Exon 21)								
1	4	2	1151.8	dPCR	560	252.33	9.75	PIK3CA (Exon 21)								
1	5	6	1789.1	dPCR	280	613.35	25.00	PIK3CA (Exon 21)								
1	6	0	3915.7	dPCR	280	62.11	0.87	PIK3CA (Exon 21)								
2	1	NA	33.6	dPCR	560	ND	ND	TP53 (Exon 7)								
2	2	NA	NA	dPCR	560	3.88	0.12	TP53 (Exon 7)								
2	3	0	25.5	dPCR	140	7.76	0.50	TP53 (Exon 7)								
2	4	0	30.7	dPCR	280	15.53	0.47	TP53 (Exon 7)								
2	5	0	32.1	dPCR	280	7.76	0.03	TP53 (Exon 7)								
3	1	10	NA	dPCR	420	150.10	2.43	PIK3CA (Exon 21)	420	5.18	0.05	PIK3CA (Exon 10)	160	1698.37	13.63	Inversion (chr 8)
3	2	4	NA	dPCR	420	56.94	1.01	PIK3CA (Exon 21)	420	ND	ND	PIK3CA (Exon 10)	96	760.87	9.38	Inversion (chr 8)
4	1	162	NA	dPCR	120	119746.38	31.87	PIK3CA (Exon 10)	28	27536.23	13.19	MET (Exon 9)	28	51319.88	37.71	IQCA1 (Exon 3)
4	2	5	NA	dPCR	80	127.91	0.42	PIK3CA (Exon 10)	232	7.80	0.05	MET (Exon 9)	232	62.35	0.47	IQCA1 (Exon 3)
4	3	0	NA	dPCR	120	ND	ND	PIK3CA (Exon 10)	232	ND	ND	MET (Exon 9)	232	7.76	0.08	IQCA1 (Exon 3)
4	4	3	NA	dPCR	120	72.46	0.74	PIK3CA (Exon 10)	186	46.74	0.47	MET (Exon 9)	186	41.41	0.55	IQCA1 (Exon 3)
4	5	71	NA	dPCR	120	4456.52	18.02	PIK3CA (Exon 10)	280	923.91	5.01	MET (Exon 9)	280	2445.65	14.92	IQCA1 (Exon 3)
4	6	2	NA	dPCR	80	644.80	2.81	PIK3CA (Exon 10)	139	141.16	0.61	MET (Exon 9)	139	218.96	1.18	IQCA1 (Exon 3)
4	7	19	NA	dPCR	210	1180.12	8.91	PIK3CA (Exon 10)	140	470.54	3.68	MET (Exon 9)	140	870.51	7.94	IQCA1 (Exon 3)
4	8	127	NA	dPCR	112	8016.30	14.24	PIK3CA (Exon 10)	56	1319.88	3.38	MET (Exon 9)	56	4968.94	12.79	IQCA1 (Exon 3)
4	9	336	NA	dPCR	7	7763.98	16.95	PIK3CA (Exon 10)	11	9523.81	7.99	MET (Exon 9)	11	17391.30	17.39	IQCA1 (Exon 3)
5	1	53	51.1	dPCR	280	1917.70	17.02	PIK3CA (Exon 10)								
5	2	25	43.0	dPCR	420	93.17	1.54	PIK3CA (Exon 10)								
5	3	31	44.5	dPCR	120	652.17	4.19	PIK3CA (Exon 10)								
5	4	54	42.2	dPCR	280	638.51	15.13	PIK3CA (Exon 10)								
5	5	78	38.7	dPCR	280	1086.96	15.45	PIK3CA (Exon 10)								
5	6	0	15.7	dPCR	420	10.35	0.32	PIK3CA (Exon 10)								
5	7	2	16.0	dPCR	280	69.88	1.57	PIK3CA (Exon 10)								
5	8	0	14.8	dPCR	280	504.66	8.72	PIK3CA (Exon 10)								
5	9	2	NA	dPCR	280	194.10	5.17	PIK3CA (Exon 10)								
5	10	13	13.5	dPCR	280	723.61	10.41	PIK3CA (Exon 10)								
5	11	7	15.9	dPCR	280	5468.13	40.84	PIK3CA (Exon 10)								
6	1	68	242.2	dPCR	280	395.96	3.94	PIK3CA (Exon 10)	160	1484.67	13.61	Deletion-1 (chr 11)	160	1970.11	17.95	Deletion-2 (chr 11)
6	2	5	184.3	dPCR	280	177.77	1.77	PIK3CA (Exon 10)	160	1046.20	14.88	Deletion-1 (chr 11)	160	1250.00	17.78	Deletion-2 (chr 11)
6	3	16	184.3	dPCR	120	634.06	1.65	PIK3CA (Exon 10)	160	5692.17	9.31	Deletion-1 (chr 11)	160	5597.63	9.22	Deletion-2 (chr 11)
6	4	6	247.3	dPCR	120	2999.55	15.40	PIK3CA (Exon 10)	160	10543.48	45.68	Deletion-1 (chr 11)	160	12581.52	54.51	Deletion-2 (chr 11)
6	5	38	798.2	dPCR	120	11231.88	27.43	PIK3CA (Exon 10)	32	78736.41	203.96	Deletion-1 (chr 11)	32	72146.74	192.39	Deletion-2 (chr 11)
7	1	0	NA	dPCR	160	ND	ND	Deletion (chr 11)	56	ND	ND	PIK3CA (Exon 21)				
7	2	NA	14.3	dPCR	320	6.79	0.26	Deletion (chr 11)	56	ND	ND	PIK3CA (Exon 21)				
7	3	NA	NA	dPCR	160	ND	ND	Deletion (chr 11)	56	ND	ND	PIK3CA (Exon 21)				
7	4	0	13.9	dPCR	160	ND	ND	Deletion (chr 11)	56	ND	ND	PIK3CA (Exon 21)				
7	5	0	16.1	dPCR	160	ND	ND	Deletion (chr 11)	56	ND	ND	PIK3CA (Exon 21)				
7	6	0	14.7	dPCR	160	ND	ND	Deletion (chr 11)	56	ND	ND	PIK3CA (Exon 21)				
8	1	0	148.9	dPCR	420	113.87	1.85	TP53 (Exon 8)								
8	2	NA	151.9	dPCR	420	124.22	1.40	TP53 (Exon 8)								
8	3	0	150.3	dPCR	420	56.94	1.51	TP53 (Exon 8)								
8	4	3	182.8	dPCR	420	284.68	4.66	TP53 (Exon 8)								
8	5	NA	NA	dPCR	210	300.21	1.63	TP53 (Exon 8)								
8	6	1	369.1	dPCR	210	1014.49	10.08	TP53 (Exon 8)								

Table S4: Serial quantification of cDNA, CA 15-3 and CTC levels during therapy

Case no.	Visit no.	CTC no. (per 7.5ml)	CA 15-3 (unit/ml)	cDNA analysis method	Volume of plasma analyzed (µl)	cDNA (Copies/ml)	Mutant allele fraction (%)	Mutation (4)	Volume of plasma analyzed (µl)	cDNA (Copies/ml)	Mutant allele fraction (%)	Mutation (5)	Volume of plasma analyzed (µl)	cDNA (Copies/ml)	Mutant allele fraction (%)	Mutation (6)
1	1	0	1293.5	dPCR												
1	2	NA	702.6	dPCR												
1	3	1	453.9	dPCR												
1	4	2	1151.8	dPCR												
1	5	6	1789.1	dPCR												
1	6	0	3915.7	dPCR												
2	1	NA	33.6	dPCR												
2	2	NA	NA	dPCR												
2	3	0	25.5	dPCR												
2	4	0	32.1	dPCR												
2	5	0	32.1	dPCR												
3	1	10	NA	dPCR	160	705.52	5.67	Translocation t(6;8)	160	353.26	4.35	Translocation t(6;8)	160	705.52	5.67	Translocation t(6;8)
3	2	4	NA	dPCR	28	46376.81	19.10	CD1A (Exon 4)	28	83876.81	27.86	KIAA0406 (Exon 3)	28	59895.65	33.75	ZFYVE21 (Exon 5)
4	1	162	NA	dPCR	232	50.82	0.44	CD1A (Exon 4)	232	27.29	0.19	KIAA0406 (Exon 3)	232	54.58	0.30	ZFYVE21 (Exon 5)
4	2	5	NA	dPCR	232	ND	ND	CD1A (Exon 4)	232	11.76	0.13	KIAA0406 (Exon 3)	232	31.06	0.21	ZFYVE21 (Exon 5)
4	3	0	NA	dPCR	186	ND	ND	CD1A (Exon 4)	232	27.29	0.39	KIAA0406 (Exon 3)	140	ND	ND	ZFYVE21 (Exon 5)
4	4	3	NA	dPCR	280	2101.45	10.86	CD1A (Exon 4)	280	4311.59	19.27	KIAA0406 (Exon 3)	280	3206.52	15.14	ZFYVE21 (Exon 5)
4	5	71	NA	dPCR	140	882.29	4.54	CD1A (Exon 4)	140	1646.90	12.39	KIAA0406 (Exon 3)	140	964.62	6.39	ZFYVE21 (Exon 5)
4	6	2	NA	dPCR	56	4231.37	29.95	CD1A (Exon 4)	56	6977.02	17.27	KIAA0406 (Exon 3)	56	7026.40	12.57	ZFYVE21 (Exon 5)
4	7	19	NA	dPCR	11	15942.03	12.79	CD1A (Exon 4)	11	31677.02	24.80	KIAA0406 (Exon 3)	7	1622.67	16.98	ZFYVE21 (Exon 5)
4	8	127	NA	dPCR												
4	9	336	NA	dPCR												
5	1	53	51.1	dPCR												
5	2	25	43.0	dPCR												
5	3	31	44.5	dPCR												
5	4	54	42.2	dPCR												
5	5	78	38.7	dPCR												
5	6	0	15.7	dPCR												
5	7	2	16.0	dPCR												
5	8	0	14.8	dPCR												
5	9	2	NA	dPCR												
5	10	13	13.5	dPCR												
5	11	7	15.9	dPCR												
6	1	68	242.2	dPCR	160	1834.24	16.71	Deletion-3 (chr 11)	160	1834.24	16.71	Deletion-3 (chr 11)	160	1834.24	16.71	Deletion-3 (chr 11)
6	2	5	188.8	dPCR	160	883.15	12.56	Deletion-3 (chr 11)	160	883.15	12.56	Deletion-3 (chr 11)	160	883.15	12.56	Deletion-3 (chr 11)
6	3	16	194.3	dPCR	160	5040.76	8.30	Deletion-3 (chr 11)	160	5040.76	8.30	Deletion-3 (chr 11)	160	5040.76	8.30	Deletion-3 (chr 11)
6	4	6	247.3	dPCR	160	19687.50	65.30	Deletion-3 (chr 11)	160	19687.50	65.30	Deletion-3 (chr 11)	160	19687.50	65.30	Deletion-3 (chr 11)
6	5	28	785.2	dPCR	32	49252.72	131.34	Deletion-3 (chr 11)	32	49252.72	131.34	Deletion-3 (chr 11)	32	49252.72	131.34	Deletion-3 (chr 11)
7	1	0	NA	dPCR												
7	2	NA	14.3	dPCR												
7	3	NA	NA	dPCR												
7	4	0	13.9	dPCR												
7	5	0	16.1	dPCR												
7	6	0	14.7	dPCR												
8	1	0	148.9	dPCR												
8	2	NA	151.9	dPCR												
8	3	0	150.3	dPCR												
8	4	2	142.3	dPCR												
8	5	NA	NA	dPCR												
8	6	1	369.1	dPCR												

Table S4: Serial quantification of ctDNA, CA 15-3 and CTC levels during therapy

Case no.	Visit no.	CTC no. (per 7.5ml)	CA15.3 (units/ml)	ctDNA analysis method	Volume of plasma analyzed (µl)	ctDNA (Copies/ml)	Mutant allele fraction (%)	Mutation (1)	Volume of plasma analyzed (µl)	cDNA (Copies/ml)	Mutant allele fraction (%)	Mutation (2)
9	1	0	26.8	gPCR	280	2795.03	27.29	PIK3CA (Exon 10)				
9	2	0	29.7	gPCR	130	286.56	2.36	PIK3CA (Exon 10)				
9	3	0	35.9	gPCR	420	36.23	0.36	PIK3CA (Exon 10)				
9	4	NA	NA	gPCR	120	60326.09	57.22	PIK3CA (Exon 10)				
10	1	2	165.7	gPCR	420	7.76	0.09	TP53 (Exon 7)				
10	2	86.0	gPCR	455	15.53	TP53 (Exon 7)	0.16	TP53 (Exon 7)				
11	1	263	86.0	gPCR	160	3500.94	36.26	Translocation (6;8)				
11	2	83	237.1	gPCR	160	2221.85	10.77	Translocation (6;8)				
12	1	0	9.6	gPCR	180	ND	ND	Deletion (chr 8)				
12	2	0	9.6	gPCR	180	ND	ND	Deletion (chr 8)				
12	3	NA	NA	gPCR	160	ND	ND	Deletion (chr 8)				
12	4	1	9.6	gPCR	160	ND	ND	Deletion (chr 8)				
12	5	0	8.1	gPCR	160	ND	ND	Deletion (chr 8)				
12	6	0	NA	gPCR	160	ND	ND	Deletion (chr 8)				
12	7	0	8.3	gPCR	160	ND	ND	Deletion (chr 8)				
12	8	NA	6.2	gPCR	160	ND	ND	Deletion (chr 8)				
13	1	111.1	gPCR	160	306.43	TP53 (Exon 7)	0.77	Translocation (1;17)				
13	2	1250	170.4	gPCR	160	95516.30	11.92	Translocation (1;17)				
14	1	1	17	gPCR	420	5.18	0.11	PIK3CA (Exon 10)				
14	2	NA	NA	gPCR	280	155.28	2.59	PIK3CA (Exon 10)				
14	3	3	22.4	gPCR	280	263.98	4.22	PIK3CA (Exon 10)				
14	4	3	28.4	gPCR	280	559.01	11.04	PIK3CA (Exon 10)				
14	5	22	46.1	gPCR	280	1141.30	13.47	PIK3CA (Exon 10)				
15	1	0	NA	TAm-Seq	200	151.20	5.35	TP53 (Exon 7)				
15	2	0	19	TAm-Seq	200	242.93	7.45	TP53 (Exon 7)				
15	3	NA	NA	TAm-Seq	200	356.30	11.90	TP53 (Exon 7)				
16	1	0	1281.6	TAm-Seq	200	53.94	0.45	TP53 (Exon 8)				
16	2	1	1950.7	TAm-Seq	200	212.17	1.60	TP53 (Exon 8)				
16	3	0	1695.9	TAm-Seq	200	481.81	5.45	TP53 (Exon 8)				
16	4	0	593.7	TAm-Seq	200	330.62	3.65	TP53 (Exon 8)				
16	5	1	NA	TAm-Seq	200	2255.13	22.15	TP53 (Exon 8)				
17	1	0	246.7	gPCR	160	27.17	0.91	Deletion (chr 8)				
17	2	NA	208.2	gPCR	160	81.52	0.38	Deletion (chr 8)				
17	3	2	121.0	gPCR	160	13.99	0.36	Deletion (chr 8)				
17	4	1	219.6	gPCR	160	176.63	1.76	Deletion (chr 8)				
17	5	3	219.4	gPCR	160	48.25	2.25	Deletion (chr 8)				
17	6	1	156.6	gPCR	160	27.17	1.19	Deletion (chr 8)				
17	7	0	118.2	gPCR	160	ND	ND	Deletion (chr 8)				
17	8	10	144.2	gPCR	160	1141.30	41.45	Deletion (chr 8)				
17	9	0	145.4	gPCR	160	95.11	0.92	Deletion (chr 8)				
18	1	3	29.2	gPCR	210	414.08	4.55	TP53 (Exon 7)				
18	2	14	NA	gPCR	105	989.65	18.89	TP53 (Exon 7)				
18	3	3	32.9	gPCR	105	3084.89	11.05	TP53 (Exon 7)				
18	4	4	22.6	gPCR	105	6632.30	36.64	TP53 (Exon 7)				
19	1	0	167.3	gPCR	280	338.42	5.92	Deletion-1 (chr 17)				
19	2	0	2317.8	gPCR	280	384.91	5.92	Deletion-1 (chr 17)				
19	3	5	3164.5	gPCR	280	3012.42	23.49	Deletion-1 (chr 17)				
19	4	0	3772.9	gPCR	280	6296.58	40.51	Deletion-1 (chr 17)				
20	1	0	9.3	TAm-Seq	200	60801.18	63.33	TP53 (Exon 6)				
20	2	0	12.1	TAm-Seq	200	170.97	3.13	TP53 (Exon 6)				
20	3	NA	14.6	TAm-Seq	200	ND	ND	TP53 (Exon 6)				
20	4	0	12.7	TAm-Seq	200	ND	ND	TP53 (Exon 6)				
20	5	0	11.1	TAm-Seq	200	ND	ND	TP53 (Exon 6)				
20	6	0	11.7	TAm-Seq	200	48.76	0.90	TP53 (Exon 6)				
20	7	0	16.7	TAm-Seq	200	ND	ND	TP53 (Exon 6)				

Table S4: Serial quantification of ctDNA, CA 15-3 and CTC levels during therapy

Case no.	Visit no.	CTC no. (per 7.5ml)	CA 15-3 (units/ml)	ctDNA analysis method	Volume of plasma analyzed (µl)	ctDNA (Copies/ml)	Mutant allele fraction (%)	Mutation (1)	Volume of plasma analyzed (µl)	ctDNA (Copies/ml)	Mutant allele fraction (%)	Mutation (2)
21	1	1	20.6	TAm-Seq	200	149.78	1.30	TP53 (Exon 5)				
21	2	38	20.2	TAm-Seq	200	542.75	1.40	TP53 (Exon 5)				
22	1	1	11.6	TAm-Seq	200	637.84	3.43	TP53 (Exon 5)				
22	2	0	11.5	TAm-Seq	200	20.75	0.73	TP53 (Exon 5)				
22	3	0	11.5	TAm-Seq	200	ND	ND	TP53 (Exon 5)				
22	4	0	10.3	TAm-Seq	200	ND	ND	TP53 (Exon 5)				
22	5	0	9.0	TAm-Seq	200	ND	ND	TP53 (Exon 5)				
23	1	72	539.4	TAm-Seq	200	1369.13	26.80	PIK3CA (Exon 21)	200	ND	ND	TP53 (Exon 8)
23	2	86	NA	TAm-Seq	200	1038.77	11.75	PIK3CA (Exon 21)	200	ND	ND	TP53 (Exon 8)
23	3	99	498.5	TAm-Seq	200	2483.10	21.35	PIK3CA (Exon 21)	200	ND	ND	TP53 (Exon 8)
23	4	116	644.5	TAm-Seq	200	710.11	14.10	PIK3CA (Exon 21)	200	ND	ND	TP53 (Exon 8)
23	5	90	700.1	TAm-Seq	200	515.54	7.65	PIK3CA (Exon 21)	200	ND	ND	TP53 (Exon 8)
23	6	72	628.1	TAm-Seq	200	543.75	10.35	PIK3CA (Exon 21)	200	14.45	0.28	TP53 (Exon 8)
24	1	103	171.8	TAm-Seq	200	17.39	1.60	TP53 (Exon 4)				
24	2	52	272.5	TAm-Seq	200	19.28	0.95	TP53 (Exon 4)				
24	3	312	580.4	TAm-Seq	200	531.59	5.60	TP53 (Exon 4)				
24	4	7	76.6	TAm-Seq	200	ND	ND	TP53 (Exon 4)				
24	5	NA	664.2	TAm-Seq	200	2183.70	8.20	TP53 (Exon 4)				
25	1	8	NA	dPCR	112	19.41	0.27	TP53 (Exon 5)				
26	1	0	26.8	TAm-Seq	200	ND	ND	TP53 (Exon 5)	200	ND	ND	PIK3CA (Exon 21)
26	2	1	30.1	TAm-Seq	200	29.59	0.83	TP53 (Exon 5)	200	ND	ND	PIK3CA (Exon 21)
26	3	0	50.9	TAm-Seq	200	35.47	1.10	TP53 (Exon 5)	200	78.00	1.65	PIK3CA (Exon 21)
27	1	8	66.7	TAm-Seq	200	109.82	14.43	TP53 (Exon 5)				
27	2	16	65.4	TAm-Seq	200	1625.13	13.72	TP53 (Exon 5)				
27	3	4	58.7	TAm-Seq	200	1352.06	29.38	TP53 (Exon 5)				
28	1	1	294.0	dPCR	560	108.70	3.54	TP53 (Exon 7)				
28	2	0	146.6	dPCR	560	3.88	0.16	TP53 (Exon 7)				
28	3	0	124.3	dPCR	560	3.88	0.15	TP53 (Exon 7)				
28	4	2	186.2	dPCR	420	56.94	2.33	TP53 (Exon 7)				
28	5	0	323.2	dPCR	280	54.35	1.43	TP53 (Exon 7)				
28	6	0	755.8	dPCR	280	135.50	0.74	TP53 (Exon 7)				
29	1	0	51.9	TAm-Seq	200	296.01	5.38	TP53 (Exon 8)				
29	2	2	70.8	TAm-Seq	200	181.91	3.33	TP53 (Exon 8)				
30	1	112	28.1	TAm-Seq	200	4488.68	14.58	TP53 (Exon 5)				
30	2	485	60.2	TAm-Seq	200	18859.42	28.60	TP53 (Exon 5)				

dPCR = digital PCR

TAm-Seq = tagged amplicon deep sequencing

ND = not detected (dPCR = no positive amplification in at least 1530 reactions, TAm-Seq = detectable mutant fraction <0.14% with <95% confidence and <10 representative reads)

Volume of plasma analyzed indicates the effective volume, calculated as the fraction of the DNA eluted from 2 ml of plasma

Table S5: Fractional mutant percentage in plasma of structural variants and point mutations in the same sample

Case no	Mutation/SV	Copy number variation	Mutant allele fraction in primary tumor (%)	Mutant percentage in plasma (%)				
				Visit 1	Visit 2	Visit 3	Visit 4	Visit 5
3	Inversion	Amplification	353.6	18.2	4.5	-	-	-
3	Translocation	Amplification	120.0	7.6	5.8	-	-	-
3	PIK3CA (Exon 21)		26.9	2.6	1.0	-	-	-
6	Deletion-1*	Amplification	141.6	18.1	4.0	15.2	37.2	73.7
6	Deletion-2*	Amplification	213.3	22.6	4.7	15.1	41.1	72.0
6	Deletion-3*	Amplification	132.2	21.4	3.4	13.8	47.8	63.7
6	PIK3CA (Exon 10)		36.2	4.0	1.0	1.7	15.4	27.4

SV = structural variant

*Deletion occurring in the setting of a complex rearrangement

Table S6: Primers and probes of digital PCR assays for quantification of somatic mutations

Case no.	Gene	Exon	Forward primer	Reverse primer	Probe 1 (VIC/HEX)	Probe 2 (FAM)	Tm
1	PIK3CA	Exon 21	AAGAGGCTTTGGAGTATTTTCATGAA	TGTTTAATTGTGGAAAGATCCAATC	CAAAATGAATGATGCACATC	TGATGCACCTTCATGGTG	60
2	TP53	Exon 7	TCCACTACAACATACATGTG	GAGTCTCCAGTGTGATG	CGGCATGAACCGGAGGCCCATC	CGGCATGAACGAGAGGCCCATC	60
3	PIK3CA	Exon 10	GCAATTTCTACACGAGATCCTCTCT	CATTTTAGCACCTTACCTGTGACTCCAT	TGAAATCACTGAGCAGGAG	TGAAATCACTAAGCAGGA	56
4	PIK3CA	Exon 21	AAGAGGCTTTGGAGTATTTTCATGAA	TGTTTAATTGTGGAAAGATCCAATC	CAAAATGAATGATGCACATC	TGATGCACCTTCATGGT	60
	PIK3CA	Exon 10	GCAATTTCTACACGAGATCCTCTCT	CATTTTAGCACCTTACCTGTGACTCCAT	TGAAATCACTGAGCAGGAG	TGAAATCACTAAGCAGGA	56
	CD1A	Exon 4	GACATCTTGCCAGTGTCTGAT	GCCTCTAGACTGCTGTGCTT	TCTCCGGCGAACC	ATCTCCGGCTAACC	60
	IQCA1	Exon 3	AAATGATATACAAACCATGTCCAGATGTATCT	AGTTGGAAGTAATAAAGGAAGAGAGAAA	CTATTCCGCTGTCCGCTA	TATTCCGCTGTCCACTA	60
	MET	Exon 9	TAGCCACCGAGAGACAAGC	CCTAATAAAGATTGGTTGGATGA	TGAAGATCTCATTGTCTAT	TGAAGATCTCATTGTCTAT	60
	ZFYVE21	Exon 5	GAGACAGCCACTATGAAATCGAAAT	GGGAAGCCTTCTGTGAGGATC	CACATTTCCACCATGC	CACATTTCCACCATGC	60
	KIAA0406	Exon 3	TATTGCACTGGGCTTTGGATT	CAATCAITGGTGGAAATGTGC	ACTCTCATCAITTTAGTTC	ACTCTCATCAITTTAGTTC	60
5	PIK3CA	Exon 10	GCAATTTCTACACGAGATCCTCTCT	CATTTAGCACCTTACCTGTGACTCCAT	TGAAATCACTGAGCAGGAG	TGAAATCACTAAGCAGGA	56
6	PIK3CA	Exon 10	GCAATTTCTACACGAGATCCTCTCT	CATTTAGCACCTTACCTGTGACTCCAT	TGAAATCACTGAGCAGGAG	TGAAATCACTAAGCAGGA	56
7	PIK3CA	Exon 21	AAGAGGCTTTGGAGTATTTTCATGAA	TGTTTAATTGTGGAAAGATCCAATC	CAAAATGAATGATGCACATC	TGATGCACCTTCATGGT	60
8	TP53	Exon 8	GGTAGGCTCCCTTTCT	TTTGAGTGGGTGTTTGTG	CGCCGGCTCTC	CGCCGGCTCTC	60
9	PIK3CA	Exon 10	GCAATTTCTACACGAGATCCTCTCT	CATTTAGCACCTTACCTGTGACTCCAT	TGAAATCACTGAGCAGGAG	TGAAATCACTAAGCAGGA	56
10	TP53	Exon 7	TGGAGTCTCCAGTGTGATGATG	CAACTACATGTGACAGTTCTGTCAT	CCTCCAGTTCATG	CCTCCAGTTCATG	60
14	PIK3CA	Exon 10	GCAATTTCTACACGAGATCCTCTCT	CATTTAGCACCTTACCTGTGACTCCAT	TGAAATCACTGAGCAGGAG	TGAAATCACTAAGCAGGA	56
18	PIK3CA	Exon 10	GCAATTTCTACACGAGATCCTCTCT	CATTTAGCACCTTACCTGTGACTCCAT	TGAAATCACTGAGCAGGAG	TGAAATCACTAAGCAGGA	56
25	TP53	Exon 7	TGTGATGATGGTGAGGATGGG	ATGTGTAACAGTTCTCTGCAATGGG	CTCCGGTTCATGC	CTCCGGATCATGCC	60
28	TP53	Exon 5	TCTACAAGCAGTCACAGC	TCACCATGCTATCTGAG	TGGGGGCAGCGCTCACAAC	TGGGGGCAGTGCCTCACAAC	60
	TP53	Exon 7	TCCACTACAACATACATGTG	GAGTCTTCCAGTGTGATG	CGGCATGAACCGGAGGCCCATC	CGGCATGAACGAGAGGCCCATC	60

Table S7: Primers of digital PCR assays for quantification of structural variants

Case no.	Rearrangement Type	Forward primer	Reverse primer
3	Translocation	CCTCATGTGACAGAAATTAAAGTATAGA	CACAAAGGTGTAGAAAAAGTTGAAG
	Inversion	TGAAAAATCCTAATATTAACCTATCAAATCA	ACATAGATGAAAAATGGGTCTGTCC
6	Deletion-1*	GCATGTGGGCCGTTTAGTAT	CCCCCATTAAGGATGCAGTA
	Deletion-2*	TTGGTACAGCTTACTCTTCCTCCT	CAGGGTGAGCTCGAAAAAAG
	Deletion-3*	GTTCTGCCTGGTAAATGATAAGA	GGTGAGGTGGATGGATCACT
7	Deletion	CGAGTCATTTCCACCACAGA	TGACACCCGTGGGTGATATTG
11	Translocation	GCTGCTGTCATTGATGCTCA	AAATCACAAAGATAGCATCATCAAA
12	Deletion	CCCAAGACCAGGCGTAAAT	TTTCCCCCTAAAAAGTGGATCG
13	Translocation	TGACAAATATCTGAATGAATCTCTAGC	TGACTTCAGCAAAACGCTCTC
17	Deletion	CCCCCAAAATTTATATGTTGAAA	TGGCAGGAGATATGGAGGAG
19	Deletion-1	GCAGGTGAAGAGTCGTGACA	TCGCTTTATTTCCCAGGTTG
	Deletion-2	TCAAAGATGTTTTTAGGTTCTTTACAT	AAGGTGTGTCAATTTGTAGCAGA

**FF = fresh frozen tissue

*Deletion occurring in setting of a complex rearrangement associated with amplification

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