LETTER

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Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA

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Cancers acquire resistance to systemic treatment as a result of clonal evolution and selection[1,2]. Repeat biopsies to study genomic evolution as a result of therapy are difficult, invasive and may be confounded by intra-tumour heterogeneity[3,4]. Recent studies have shown that genomic alterations in solid cancers can be characterized by massively parallel sequencing of circulating cell-free tumour DNA released from cancer cells into plasma, representing a non-invasive liquid biopsy[5–7]. Here we report sequencing of cancer exomes in serial plasma samples to track genomic evolution of metastatic cancers in response to therapy. Six patients with advanced breast, ovarian and lung cancers were followed over 1–2 years. For each case, exome sequencing was performed on 2–5 plasma samples (19 in total) spanning multiple courses of treatment, at selected time points when the allele fraction of tumour mutations in plasma was high, allowing improved sensitivity. For two cases, synchronous biopsies were also analysed, confirming genome-wide representation of the tumour genome in plasma. Quantification of allele fractions in plasma identified increased representation of mutant alleles in association with emergence of therapy resistance. These included an activating mutation in *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha) following treatment with paclitaxel[8]; a truncating mutation in *RB1* (retinoblastoma 1) following treatment with cisplatin[9]; a truncating mutation in *MED1* (mediator complex subunit 1) following treatment with tamoxifen and trastuzumab[10,11], and following subsequent treatment with lapatinib[12,13], a splicing mutation in *GAS6* (growth arrest-specific 6) in the same patient; and a resistance-conferring mutation in *EGFR* (epidermal growth factor receptor; T790M) following treatment with gefitinib[14]. These results establish proof of principle that exome-wide analysis of circulating tumour DNA could complement current invasive biopsy approaches to identify mutations associated with acquired drug resistance in advanced cancers. Serial analysis of cancer genomes in plasma constitutes a new paradigm for the study of clonal evolution in human cancers.

Serial sampling of the tumour genome is required to identify the mutational mechanisms underlying drug resistance[2]. Serial tumour biopsies are invasive and often unattainable. Tumours are heterogeneous and continuously evolve, and even if several biopsies are obtained, these are limited both spatially and temporally. Analysis of isolated circulating tumour cells (CTCs) has been proposed, but circulating tumour DNA (ctDNA) is more accessible and easier to process[15]. Previous studies of tumour mutations in plasma have analysed individual loci, genes or structural variants to quantify tumour burden and to detect previously-characterized resistance-conferring mutations[1,6,16–18]. Genome-wide sequencing of plasma samples is used in prenatal diagnostics, demonstrating comprehensive coverage of the genome[19]. More recently, genome-wide sequencing of plasma DNA has been demonstrated as a potential tool for detection of disease or analysis of tumour burden in patients with advanced cancers[5,7]. These studies established that plasma DNA contains representation of the entire tumour genome[7], mixing together variants originating from multiple independent tumours[5]. This suggests that deeper sequencing of plasma DNA, applied to selected samples with high tumour burden in blood, may allow assessment of clonal heterogeneity and selection. In this study, we applied exome sequencing of ctDNA as a platform for non-invasive analysis of tumour evolution during systemic cancer treatment (Fig. 1).

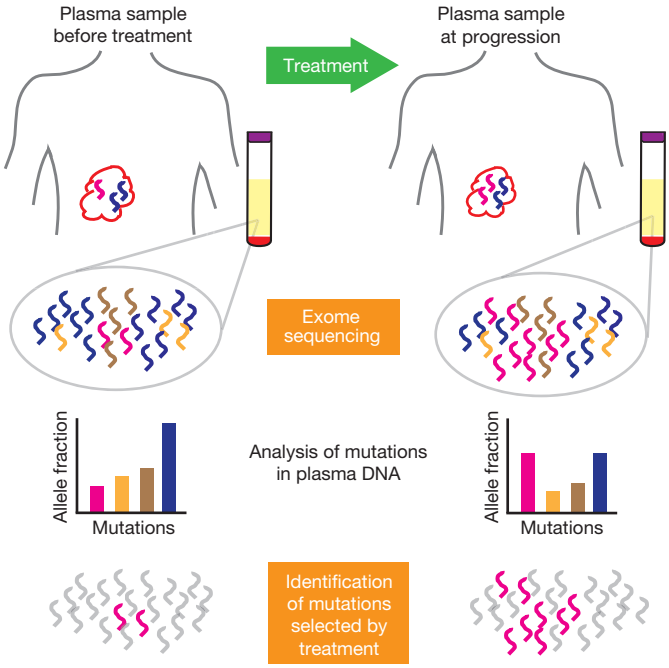

Figure 1 | Identification of treatment-associated mutational changes from exome sequencing of serial plasma samples. Overview of the study design: plasma was collected before treatment and at multiple time-points during treatment and follow-up of advanced cancer patients. Exome sequencing was performed on circulating DNA from plasma at selected time-points, separated by periods of treatment, and germline DNA. Mutations were identified across the plasma samples, and their abundance (allele fraction) at different time-points compared, generating lists of mutations that showed a significant increase in abundance, which may indicate underlying selection pressures associated with specific treatments. These lists contained mutations known to promote tumour growth and drug resistance, but also mutations of unknown significance. Accumulating such data across large cohorts could identify genes or pathways with recurrent mutations.

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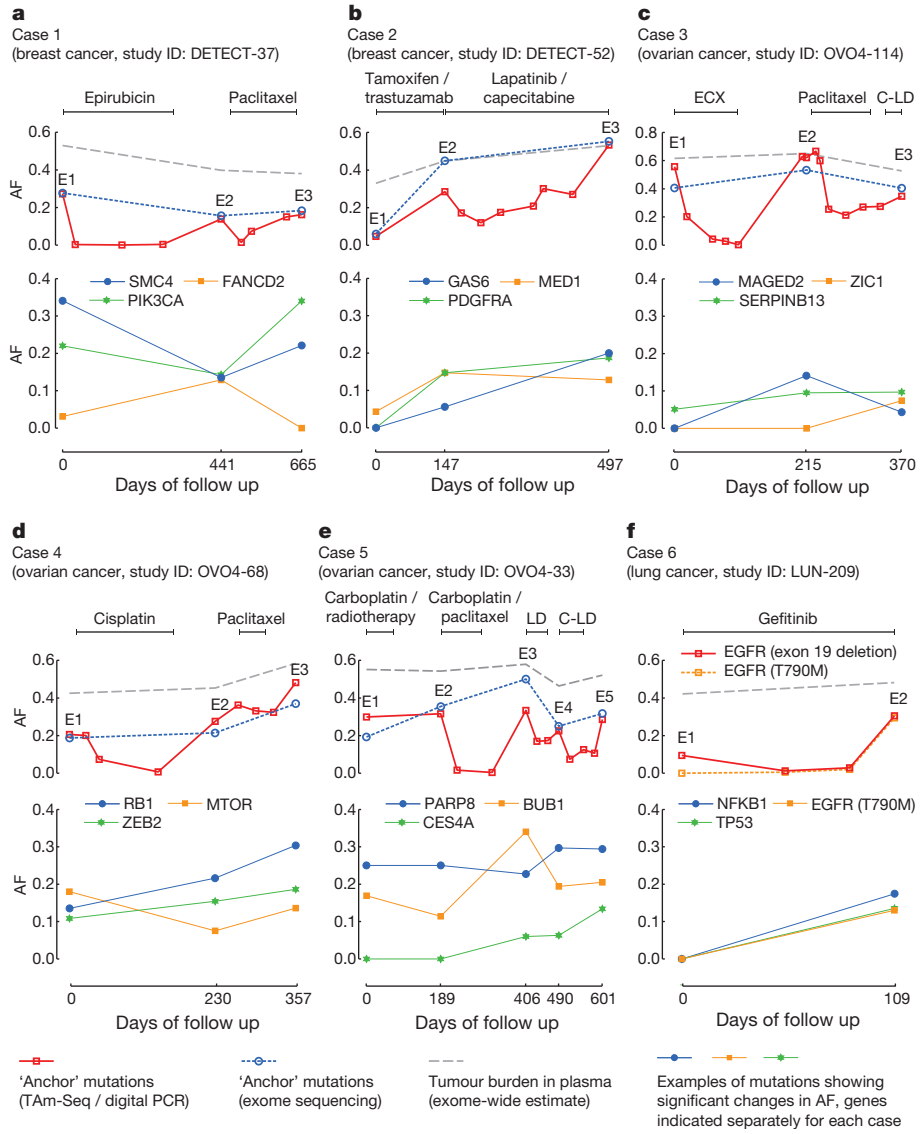

Figure 2 | Mutations showing evidence of genomic tumour evolution. All panels (**a**–**f**) are made up of an upper and a lower subpanel. Upper subpanels, time courses for allele fractions (AF; data points) of 'anchor' mutations used for initial quantification of ctDNA levels, and the fractional concentration of tumour DNA (tumour burden; grey dashed lines). 'Anchor' mutations were measured using digital PCR or TAm-Seq[6] for all available plasma samples, and using exome sequencing at selected time points indicated by E1, E2, E3 (and E4 and E5 for case 5). Tumour burden was estimated from exome data (an adaptation of genome-wide aggregated allelic loss[7]). In **a**, AF was averaged over six mutations measured in parallel using digital PCR. In **b**, a single mutation in *ATM* (predicted amino acid change I2948F) was measured by TAm-Seq. In **c**, **d** and **e**, a single mutation in *TP53* was measured by digital PCR for each case (R175H, K132N and R175H, respectively). In **f**, digital PCR was used to measure abundance of a deletion in exon 19 of *EGFR* (not quantified in exome sequencing data) and the EGFR T790M mutation. Lower subpanels, AF in exome data for selected mutations (blue, green and orange datapoints, see key) for each of the cases. Additional details are listed in Table 1, and a full list of mutations that showed a significant increase in abundance is included in Supplementary Tables 2–7. ECX, epirubicin, cisplatin and capecitabine; C-LD, carboplatin and liposomal doxorubicin; LD, liposomal doxorubicin.

We performed whole exome sequencing of plasma DNA in six patients with advanced cancers (Supplementary Table 1): two with breast cancer (cases 1 and 2), three with ovarian cancer (cases 3–5), and one with non-small-cell lung cancer (NSCLC, case 6). Exome sequencing was performed on multiple plasma samples from each patient separated by consecutive lines of therapy, spanning up to 665 days of clinical follow up (range 109–665 days, median 433 days). The ability to detect genomic events using redundant sequencing is dependent on the allele fraction (AF) of the mutant alleles in the samples analysed (ratio of mutant reads to depth of coverage at that locus), the sequencing depth, and the background noise rates of sequencing. Levels of ctDNA were previously quantified in these patients using digital PCR and tagged-amplicon deep sequencing[6] to the tumour could be identified even at relatively modest depth of sequencing. Comparison of AF measured using exome sequencing, digital PCR and TAm-Seq showed a high degree of concordance (correlation coefficient 0.8, $P < 0.0001$; Supplementary Fig. 1). Using as little as 2.3 ng of DNA (4%–20% of the DNA extracted from 2.0–2.2 ml of plasma), and an average of 169 million reads of sequencing per sample, we analysed the coding exons of all protein-coding genes at an average unique coverage depth ranging from 31-fold to 160-fold across 19 plasma samples (Supplementary Table 2). Consistent with previous reports[5,7], we observed copy number aberrations (CNAs, both gains and losses) in plasma samples in all patients across the whole genome (Supplementary Figs 2–7). These were strongly modulated by the fraction of tumour DNA in plasma and

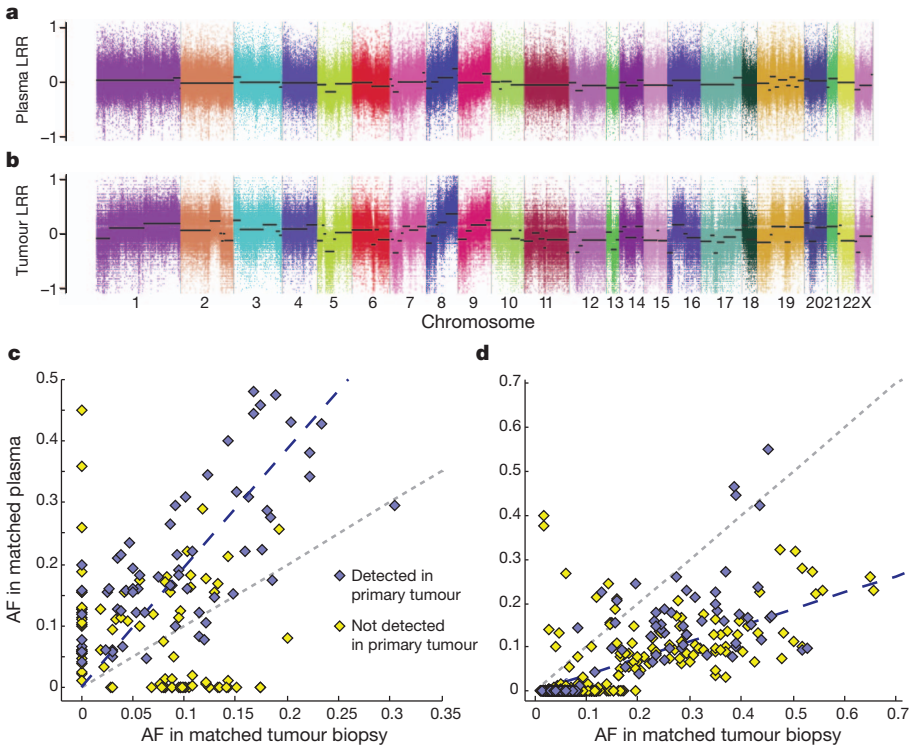

Figure 3 | Genome-wide concordance between plasma DNA and tumour DNA. **a**, **b**, Sequencing data were used to assess CNAs in the plasma sample (**a**) and in the synchronous metastatic tumour biopsy (**b**) from case 4. Panels show log *R* ratio (LRR), calculated on the basis of exome data, between plasma DNA and normal DNA (**a**) and between tumour and normal DNA (**b**). **c**, AF of mutations identified in exome data from plasma or metastatic biopsy for case 1. Grey dotted line shows equality. Blue dashed line has a slope of 1.93, indicating the median of the AF ratio for mutations found in both samples. Key applies to **c** and **d**. **d**, As **c** but for case 4, blue dashed line has a slope of 0.37.

For two cases, sequencing data were also available from metastatic tumour biopsies, collected at the same time as plasma samples (case 1 sample E1, and case 4 sample E2), and from tumour samples collected at the patients' initial presentation, 9 and 4.5 years earlier. CNAs were concordant between plasma and metastasis DNA in both patients (Fig. 3a, b, and Supplementary Fig. 7). Mutations identified in sequencing data[20–23] from the plasma or metastatic biopsy were compared (Supplementary Information). In case 1 with breast cancer, 151 mutations were identified in either the plasma or the synchronous biopsy. Of these, 93 mutations were found in both, and mutant AFs for these were higher in the plasma sample compared to the metastatic biopsy. The correlation coefficient of mutant AFs was positive (0.71) for mutations that were also found in the primary tumour, but negative (−0.22) for other mutations (Fig. 3c). In case 4 with ovarian cancer, 895 mutations were identified in either plasma or the tumour biopsy. For 172 mutations found in both, AFs were positively correlated (0.72) and were higher in the metastatic biopsy, which also contained 686 'private' mutations with AF < 0.2 that were not found in either the plasma or the earlier tumour sample (Fig. 3d).

To identify changes in the mutation profiles of the tumours, we compared the abundance of somatic mutations found in plasma before and after each course of systemic treatment. For each patient, we examined a conservative list of mutations, including all mutations that were called in any of the plasma samples with a Bonferroni-corrected binomial probability of <0.05 assuming a background sequencing error rate of 0.1%. For each mutation and course of treatment (spanned by a pair of plasma samples), a *P*-value for a possible change in mutant AF was calculated as the binomial probability of obtaining the observed number of mutant reads, given the sequencing depth and the observed abundance in the paired time-point, normalized by the fractional concentration of tumour-derived DNA in the plasma (based rate of <10% for significant changes in normalized abundance, ranging from 15 to 121 for each case (median 49). These include mutations in well-known cancer genes, genes linked to drug resistance and drug metabolism, and genes not previously associated with carcinogenesis or therapy resistance (Supplementary Tables 4–9). Selected examples are shown in Table 1 and Fig. 2.

We highlight here five examples. In case 1 with breast cancer, a strong increase was observed in the abundance of an activating mutation in *PIK3CA* following treatment with paclitaxel (Fig. 2a and Table 1). This mutation has been shown to promote resistance to paclitaxel in mammary epithelial cells[8]. In case 2, a patient with an oestrogen-receptor (ER)-positive, HER2-positive breast cancer, treatment with tamoxifen in combination with trastuzumab led to an increase in abundance of a nonsense mutation near the carboxy terminus of *MED1*, an ER co-activator that has been shown to be involved in tamoxifen resistance[10,11]. After further treatment of this patient with lapatinib in combination with capecitabine, we observed an increase in abundance of a splicing mutation in *GAS6*, the ligand for the tyrosine kinase receptor AXL (Fig. 2b, Table 1). Activation of the AXL kinase pathway has been shown to cause resistance to tyrosine kinase inhibitors in NSCLC[13] and resistance to lapatinib in ER-positive, HER2-positive breast cancer cell lines[12]. In case 4 with ovarian cancer, following treatment with cisplatin, we observed increase in abundance of a truncating mutation in the tumour-suppressor *RB1* (Fig. 2d, Table 1), predicted to inactivate the RB1 protein (Supplementary Fig. 8). In the matched metastasis biopsy obtained after treatment, the mutation was found in 95% of sequencing reads (59 of 62), with apparent loss of heterozygosity at 13q containing the *RB1* gene (Fig. 3a, b). Loss of *RB1* has been linked with chemotherapy response[9]. Case 6 was a NSCLC patient with an activating mutation in *EGFR* who was treated with gefitinib but progressed on treatment. Analysis by digital

Table 1 | Selected mutations whose mutant AF significantly increased following treatment

Patient	Cancer type	Gene	Effect	Potential biological interest	Associated treatment	Mutant AF in plasma	
						Before	After
Case 1	Breast	*PIK3CA*	E545K	PI-3-kinase. p.E545K mutation associated with chemoresistance in mammary epithelial cells[8].	Paclitaxel	14%	34%
Case 1	Breast	*BMI1*	S324Y	BMI1 polycomb ring finger oncogene. Associated with chemoresistance[25].	Paclitaxel	3%	12%
Case 1	Breast	*SMC4*	I1000S	Structural maintenance of chromosomes 4. Downregulated in taxane resistant cell lines[26].	Paclitaxel	14%	22%
Case 1	Breast	*FANCD2*	G56V	Fanconi anaemia complementation group D2. Chromatin dynamics and DNA crosslink repair[27].	Epirubicin	3%	13%
Case 2	Breast	*MED1*	S1179X	Mediator complex subunit 1. Co-activator of ER with functional role in tamoxifen resistance[10,11].	Tamoxifen/trastuzumab	4%	15%
Case 2	Breast	*ATM*	I2948F	Ataxia telangiectasia mutated.	Tamoxifen/trastuzumab	6%	45%
Case 2	Breast	*PDGFRA*	D714E	Platelet-derived growth factor alpha. Cell surface tyrosine kinase receptor.	Tamoxifen/trastuzumab	0%	15%
Case 2	Breast	*GAS6*	Splicing	Growth arrest-specific 6. Ligand for AXL, overexpression associated with TKI resistance[12,13].	Lapatinib/capecitabine	6%	30%
Case 2	Breast	*TP63*	Splicing / S551G	Tumour protein p63.	Lapatinib/capecitabine	4%	20%
Case 4	Ovarian	*RB1*	E580X	Retinoblastoma 1. Loss of RB1 associated with EMT and drug resistance[9].	Cisplatin	14%	22%
Case 4	Ovarian	*ZEB2*	Y663C	Zinc finger E-box binding homeobox 2. Overexpression associated with cisplatin resistance in ovarian cancer[28].	Cisplatin	11%	15%
Case 4	Ovarian	*MTOR*	K1655N	Mechanistic target of rapamycin. Activating mutations in mTOR confers resistance to antimicrotubule agents[29].	Paclitaxel	8%	14%
Case 5	Ovarian	*CES4A*	P55S	Carboxylesterase 4A. Hydrolysis or transesterification of various xenobiotics.	Carboplatin/paclitaxel Carboplatin/liposomal doxorubicin	0% 6%	6% 13%
Case 5	Ovarian	*BUB1*	M889K	Mitotic checkpoint serine/threonine-protein kinase.	Carboplatin/paclitaxel	11%	34%
Case 5	Ovarian	*PARP8*	P81T	Poly [ADP-ribose] polymerase family, member 8.	Liposomal doxorubicin	23%	30%
Case 6	Lung	*EGFR*	T790M	Epidermal growth factor receptor. Established to cause gefitinib resistance by inhibiting drug binding[14].	Gefitinib	0%	13%
Case 6	Lung	*TP53*	Y163C	Tumour protein p53[30].	Gefitinib	0%	14%
Case 6	Lung	*NFKB1*	G489V	Nuclear factor κB[30].	Gefitinib	0%	17%

Potential biological role and associations with drug resistance described in literature are highlighted. The ''Effect'' column lists predicted change in amino acid sequence.

gefitinib to EGFR and has been established as the main driver of acquired resistance to gefitinib[14]. Unbiased analysis of plasma DNA by exome sequencing identified selection for this mutation amongst genomic changes that occurred following therapy (Fig. 2f, Table 1).

In this proof of principle study, we demonstrate that exome analysis of plasma ctDNA represents a novel paradigm for non-invasive characterization of tumour evolution. Our data, together with recent reports[5,7], show that CNAs and somatic mutations identified in ctDNA are widely representative of the tumour genome and provide an alternative method of tumour sampling that can overcome limitations of repeated biopsies. Cell-free DNA fragments from multiple lesions in the same individual all mix together in the peripheral blood[5], therefore ctDNA is likely to contain a wider representation of the genomes from multiple metastatic sites, whereas mutations present in a single biopsy or minor sub-clone may be missed. This strengthens the case for the use of ctDNA as a biomarker for monitoring tumour burden or for the analysis of hotspot mutation regions[1,6,16,17], but also indicates that tracking different mutations for assessment of tumour heterogeneity and clonal evolution is now possible. Our data identified a subset of genes that were positively selected following treatment, many of which have been previously associated with drug resistance. Other changes may represent 'passenger' mutations or false-positives, but some are likely to contribute to resistance to therapy. Accumulating data across a large number of cases could identify new genes or pathways that are frequently mutated following specific treatment types, and help refine analysis algorithms.

The approach we describe here may be broadly applicable to a large fraction of advanced cancers, where the median mutation burden in plasma (before start of treatment) is 5%–10% (refs 6, 16, 24). Analysis of acquired drug resistance is of particular utility in advanced or metastatic cancers, which is the target population for nearly all early phase clinical trials. Improvements in sequencing and associated technologies may enable similar analysis in cases with a lower tumour burden in plasma burden, enabling detailed and comprehensive evaluation of clonal genomic evolution associated with treatment response and resistance.

METHODS SUMMARY

Patients and samples. Cases 1–5 were recruited as part of prospective clinical studies at Addenbrooke's Hospital, Cambridge, UK, approved by the local research ethics committee (REC reference nos 07/Q0106/63, 08/H0306/61 and 07/Q0106/63). Case 6 was recruited as part of the 'Hydroxychloroquine and gefitinib to treat lung cancer' study (NCT00809237) at the National University Health System, Singapore, approved by the National Healthcare Group NHG IRB—DSRB 2008/00196. Written informed consent was obtained from patients, and serial blood samples were collected at intervals of ≥3 weeks.

Extraction and sequencing of plasma DNA. DNA was extracted from plasma using the QIAamp circulating nucleic acid kit (Qiagen) according to the manufacturer's instructions. Barcoded sequencing libraries were prepared using a commercially available kit (ThruPLEX-FD, Rubicon Genomics). Pooled libraries were enriched for the exome using hybridization (TruSeq Exome Enrichment Kit, Illumina), quantified using quantitative PCR and pooled in 1:1 ratio for paired-end sequencing on a HiSeq2500 (Illumina).

Variant calling and analysis. Sequencing data were demultiplexed and aligned to the hg19 genome using BWA[20]. Pileup files for properly paired reads with mapping quality ≥60 were generated using samtools[22]. AFs were calculated for all Q30 bases. A mutation was called if ≥4 mutant reads were found in plasma with ≥1 read on each strand, and no mutant reads were observed in germline DNA or in a prior plasma sample with ≥10-fold coverage. For comparison between consecutive plasma samples in a patient, we calculated the binomial probability of obtaining the observed AF (or greater) if the abundance of the mutant allele, normalized by tumour load in plasma (based on a modified genome-wide aggregated allelic loss method[5]), had remained constant between the two samples.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions M.M., S.-J.D., T.F., D.W.Y.T., D.G., J.D.B., C.C. and N.R. designed the study. M.M., D.W.Y.T. and T.F. developed methods. S.-J.D., C.P., A.S.C.W., T.M.C., J.D.B. and C.C. designed and conducted the prospective clinical studies. M.M., S.-J.D., D.W.Y.T., D.G., T.F. and A.M.P. generated data. Z.K., S.H. and D.B. contributed sequencing data. M.M., F.M. and N.R. analysed sequencing data. S.-F.C. and J.H. contributed to experiments and data analysis. M.M., S.-J.D., D.W.Y.T., T.M.C., J.D.B., C.C. and N.R. interpreted data. M.M. and N.R. wrote the paper with assistance from S.-J.D., D.W.Y.T., C.C., J.D.B. and other authors. All authors approved the final manuscript. J.D.B., C.C. and N.R. are the project co-leaders and joint senior authors.

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