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Genomic alterations in circulating tumor DNA from diverse cancer patients identified by next-generation sequencing

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Abstract

Non-invasive genomic profiling of tumors may be possible with next-generation sequencing (NGS) of blood-derived circulating tumor DNA (ctDNA), but proof of concept in a large cohort of patients with diverse cancers has yet to be reported. Here we report the results of an analysis of plasma-derived ctDNA from 670 patients with diverse cancers.

The tumors represented in the patient cohort were mainly gastrointestinal (31.8%), brain (22.7%) or lung (20.7%). ctDNA obtained from most patients (N = 423 (63%)) displayed at least 1 alteration. The most frequent alterations seen, as characterized mutations or variations of unknown significance, occurred in *TP53* (32.5% of patients), *EGFR* (13%), *KRAS* (12.5%) and *PIK3CA* (9.1%); for characterized alterations, 30.7% (*TP53*), 7.6% (*EGFR*), 12.2% (*KRAS*), and 7.7% (*PIK3CA*). We found that 32% of brain tumors had at least 1 ctDNA alteration. Head and neck tumors were independently associated with a higher number of alterations in a multivariable analysis (P=0.019). Notably, 320/670 (48%) of patients displayed potentially actionable alterations with 241 patients possible candidates for on-label or off-label treatment with an FDA-approved drug. Several illustrations of the clinical utility of the information obtained for improving treatment of specific patients is provided. Our findings demonstrate the feasibility and impact of genomic profiling of tumors by ctDNA next-generation sequencing, greatly encouraging broader investigations of the application of this technology for precision medicine in cancer management.

Keywords

Cancer; liquid biopsy; ctDNA; actionable alteration; personalized therapy

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Introduction

The landscape of the cancer genome features frequently altered genes that are potential clinical targets. Technology to detect circulating tumor DNA (ctDNA) derived from blood (liquid biopsy) is a promising tool to interrogate the molecular characteristics of tumors. The discipline of noninvasive disease monitoring has advanced greatly since cell-free DNA (cfDNA) was first reported in body fluids. In contrast to liquid biopsies, the invasive nature of tissue biopsies poses a risk to patients and can be costly. Tumor sampling for some cancer types is difficult and, as a result, inadequate amount or quality of tissues is often an issue. Indeed, in metastatic non-small cell lung cancers (NSCLC), almost 30% of patients do not have easily accessible tissue (1). In addition, tissue preservation methods such as formalin fixation can cause cytosine to thymine transitions by cytosine deamination, theoretically leading to false-positive results during sequencing (2). Finally, due to tumor heterogeneity, biopsies suffer from their limitation of reflecting only the small piece of tissue sampled (3,4).

The tumor ecosystem is dynamic and is able to change its dominant mutation pattern or acquire new alterations in response to selective pressure from previous therapies. Tumor evolution is of specific concern when stratifying subjects to a specific targeted therapy based on a mutation profile from an old report. For example, approximately 50% of patients with NSCLC become resistant to initial tyrosine kinase inhibitor therapy through an EGFR T790M mutation (5).

Tumor cells often shed their DNA content upon cell death. The result is that there is often ctDNA detectable in the bloodstream. Interestingly, it has been observed that patients with more advanced disease have higher concentrations of ctDNA, and that ctDNA percentage correlates with survival (6). While detection of ctDNA is still challenging since only a very small fraction of total cell-free DNA is derived from tumors (7), next-generation sequencing (NGS) has made analysis more feasible. Indeed, it is now becoming possible to map the genomic makeup of a cancer and identify potential treatment options with just a simple blood test (8,9). ctDNA assessment often (though not always) shows high overall agreement with tissue genomic tests (10) and may be an attractive technology to serially monitor cancer progression (8,9,11,12).

To facilitate studies that explore the utility of ctDNA analysis, a comprehensive understanding of the findings in diverse cancer types is essential.

Herein, we report the results of digital NGS ctDNA analysis in 670 patients. Most patients demonstrated genomic alterations in their ctDNA. We also describe three illustrative patients (gastric and lung cancer) who had *EGFR* ctDNA alterations and achieved partial responses after EGFR-targeted therapy, and a third patient (colon cancer) in whom *EGFR* and *KRAS* resistant alterations in ctDNA emerged with acquired resistance after initial response on cetuximab.

Materials and Methods

Patients

We retrospectively reviewed the liquid biopsy results of 670 consecutive, de-identified patients with diverse cancers who were seen at the University of California San Diego (UCSD), Moores Cancer Center. Pathology was reviewed at UCSD. Blood samples were collected between June 4th, 2014 and December 17th, 2015. This study was performed in compliance with UCSD IRB exempt approval for study of pre-existing de-identified data and the Declaration of Helsinki. [Patient case studies gave consent (NCT02478931)]. Analysis of usage patterns of liquid biopsies at our institution indicates that the majority of patients who have had these tests performed have advanced or metastatic disease.

Sequencing

Digital Sequencing was performed by Guardant Health, Inc. (Guardant360, www.guardanthealth.com/guardant360/), a Clinical Laboratory Improvement Amendment (CLIA)-certified and College of American Pathologists (CAP)-accredited clinical laboratory. For 150 patients, a 54 gene panel was used. This test identifies potential tumor-related alterations within 54 cancer-related genes (Supplemental Table 1) including amplifications in *ERBB2*, *EGFR*, and *MET* through analysis of cell-free DNA extracted from plasma (from two 10 ml blood tubes; indels and fusions were not detected as part of this panel)(13). For 400 patients, a 68-gene version of the original panel (expanded to all four major alteration types) was used, and for 120 patients, the most recent 70 gene panel version (further expanded to amplifications in 18 genes and fusions in 6 genes) was applied (Supplemental Tables 2 and 3). Only non-synonymous alterations were analyzed in our study (variants of unknown significance (VUSs) did not include synonymous alterations). Alterations were dichotomized into “characterized” or “VUS” at the variant level.

The ctDNA assay used in this study has a high clinical sensitivity (detects 85%+ of the single nucleotide variants detected in tissue in advanced cancer patients) and analytic specificity (>99.9999%). A high degree of specificity is critical to eliminate the false positives (noise) that otherwise accompany sequencing DNA at very low concentrations over long targeted regions. All cell-free DNA is sequenced, including the germline cell-free DNA that is derived from leukocyte lysis and the somatic ctDNA. Only non-germline somatic alterations were reported and analyzed in this study (Guardant360 is currently not validated to report germline alterations). Single nucleotide variants are quantitated as mutant allele fraction (MAF), which is the number of ctDNA fragments, divided by the number of wild type DNA fragments that overlap the same mutated nucleotide base position. Gene amplifications are reported as absolute gene copy number in plasma. In each sequencing run, a normal control sample is included (Guardant360 digital sequencing panel, Guardant Health Inc, data on file).

Potential actionability

Actionability implies that the protein product of a genomic abnormality can potentially be impacted by a specific targeted drug (14). A potentially actionable alteration was defined as a characterized alteration that was either the direct target (such as an EGFR inhibitor

targeting an *EGFR* mutation), or a pathway component (such as an mTOR inhibitor for a *PIK3CA* mutation (since mTOR is downstream of *PIK3CA*)) that could be targeted by at least one approved (by the Food and Drug Administration–FDA) or investigational drug in a clinical trial. Potential actionability was crosschecked by two investigators; including the senior investigator (RK).

Data extraction and analysis

Demographic information such as gender, age, primary tumor site, as well as the dates of sample collection, test results, list of actionable alterations data (the number of alterations with an approved drug available in the disease (on-label use), the number of alterations with an approved drug in another disease (off-label use), and the number of alterations with experimental drug(s) available (clinical trials) were extracted from the reports and analyzed. When appropriate, median and 95% confidence intervals (95% CI) or range were reported. The sample size was determined by the available patients with genetic testing information at the time of analysis, and most of the statistical analysis (performed by MS; program: SPSS version 24.0) was descriptive in nature.

Results

Patient characteristics

Our population included 670 patients with diverse cancers who had a biopsy-free digital NGS ctDNA test performed on their plasma. Patient's median age was 62 years old (Range, 5–92). Women and men were well balanced, with about 50% in each group. The most common tumor sites were gastrointestinal (31.8%), followed by brain (22.7%) and lung (20.7%) (Table 1).

Circulating tumor DNA (ctDNA) sequencing results

The median testing turnover time, defined as the median time from sample collection until results, was 15 days (95%CI, 15–16 days; range 7–35 days). In the overall population (670 tested patients), 63.1% of patients had at least one alteration detected in their plasma (Table 1 and Figure 1). The most frequent alterations identified were *TP53* (32.5%), followed by *EGFR* (13.0%), *KRAS* (12.5%), *PIK3CA* (9.1%) and *APC* (6.6%) (Figure 2A), and the median percentage of ctDNA detected for all the alterations (N=1,387) in our population was 0.98% (Figure 2B). Of interest, 32% of brain tumors (N = 49/152) had at least one alteration detected in their plasma. Head and neck cancers comprised the tumor type with the highest percentage of patients with alterations detected (88%), as well as the highest frequency of patients with more than three alterations detected (48%) (Figure 1 and 2C). This was confirmed by a multivariable analysis (linear regression model) looking at the correlation between patient's clinical characteristics and the number of detected alterations; head and neck tumors were an independent predictor of a higher number of alterations (P=0.019, median of 3 alterations (95%CI 1–6)), while brain tumors correlated with less alterations (P=0.017, median of 0 alterations identified) (Table 2). The examination of characterized alterations (excluding VUSs) also concluded that head and neck tumors were associated with a statistically higher number of characterized alterations (P=0.008, median 2 (95%CI 1–4) in a multivariable analysis) (Table 2 and Figure 1). In addition, the presence of

either *TP53*, *EGFR*, *KRAS*, and/or *PIK3CA* alteration(s), as well as the detection of at least one alteration with a ctDNA percentage $\geq 10\%$ were also statistically independent predictors of an increased number of alterations detected in patient's plasma (all $P < 0.001$ in the multivariable analyses when both "all alterations" or "characterized alterations only" considered, Table 2).

Actionability of the detected alterations

Of the total 670 analyzed patients, 320 had characterized, potentially actionable alterations (48% of total, 76% of the patients with alterations detected), Suppl Table 4. Indeed, amongst these 320 patients, 241 patients (75% of 320 or 36% of total patients) had at least one potentially matched FDA-approved drug as a treatment option (N=45 on-label and N=196 off-label only), Figure 3A. Of note, the majority of patients with gastrointestinal, lung, breast, and head and neck cancers had hypothetically actionable alterations (56%, 67%, 62%, and 76%, respectively), Figures 3B and 3C.

On the other hand, 350 patients (52% of total) had no actionable alterations. This was because no alterations were detected by the ctDNA test (247 patients, 37% of total) or because the alterations detected were variants of unknown significance (101 patients, 24%), Figure 3A.

Case Study 1: A patient with gastric cancer and *EGFR* amplification on ctDNA treated with anti-*EGFR* therapy—

A 68-year-old woman presented to the clinic with advanced gastric cancer and liver metastasis. Tissue NGS (315 genes) revealed multiple amplifications including in *EGFR*, *CCND3*, *MAP2K1*, *VEGFA*, *AURKA*, *ARFRP1*, *GNAS*, *PIK3CG* and *ZNF217* genes, as well as a *TP53* G245S mutation. ctDNA analysis also revealed *EGFR* amplification. Since *EGFR* amplification was identified in both tissue and ctDNA profiling, the patient was started on an anti-*EGFR*-based regimen, which included both erlotinib and cetuximab (15). One week after the initiation of anti-*EGFR* therapy, the patient noticed significant improvement in her abdominal fullness and pain. Re-staging imaging approximately two months after the initiation of therapy showed reduction of multiple liver metastases (overall 40% decrease by RECIST 1.1. Response is ongoing at the most recent restaging (60% reduction at 5 months) (Figure 4A). Along with the reduction of tumor burden, *EGFR* copy number in blood circulation rapidly declined (originally 143.9, down to reference level 2.0) (Figure 4B). Carcinoembryonic antigen, which was originally 101 ng/ml also decreased to undetectable levels.

Case Study 2: A patient with adenocarcinoma of the lung and an *EGFR* ctDNA mutation treated with erlotinib—

A 77-year-old woman was diagnosed with adenocarcinoma of the lung and multiple brain and bone metastases. ctDNA revealed an *EGFR* del 19 (E746_A750 del) mutation. Molecular tissue NGS analysis later confirmed *EGFR* E746_A750del, and also showed *EGFR* amplification, *AURKA* amplification, *GNAS* amplification, *TP53* splice site 375+1G >T, and *ZNF217* amplification. The patient was started on erlotinib and was expected to begin brain radiation. A week into the erlotinib treatment, brain imaging performed as a part of radiation treatment planning showed near complete resolution of all brain metastases, and radiation treatment plans were aborted.

Follow-up chest computerized tomography showed partial response (Figure 4C). Eight months later, imaging indicated progression. Repeat ctDNA analysis demonstrated the original *EGFR* E746_A750 del mutation, *EGFR* amplification and no *EGFR* T790M secondary resistance mutation. Molecular analysis from a post-progression biopsy revealed, *EGFR* E746_A750del and *TP53* splice site 375+1G >T. Because of the lack of *EGFR* T790M resistance mutation in either ctDNA or tissue, chemotherapy was initiated.

Case Study 3: Patient with *K-RAS* wild-type colon cancer treated with cetuximab-based therapy. ctDNA analysis at progression with multiple resistance alterations—A 55-year-old man with metastatic colon cancer was initiated on third-line therapy with cetuximab (anti-EGFR antibody) based therapy, along with 5-fluorouracil and irinotecan, after tumor progression on an oxaliplatin, irinotecan and bevacizumab based regimen. Biopsy of the liver mass performed one month after the initiation of cetuximab-based therapy for tissue NGS revealed *TP53* R282W and *APC* Y935fs*1 alterations. The patient initially attained a partial response to the cetuximab-based regimen; however, the tumor gradually progressed after a year of therapy (Figure 4D). ctDNA analysis at the time of progression revealed multiple new alterations including *EGFR/ERBB2/KRAS/PIK3CA* amplifications, *EGFR* S492R, *BRAF* G469A, *KRAS* G12A/G12C/G13D/Q61H and *NRAS* Q61H alterations (Figure 4D).

Discussion

In this large study, we describe the molecular alterations identified in 670 patients suffering from various cancers. Targeted NGS that analyzed ctDNA from plasma was utilized. In the overall population, 63% of patients had at least one molecular alteration (in 48% of patients, it was a characterized alteration and, in the others, only a VUS). Consistent with our previous study (8), the most frequent alterations were in the *TP53* tumor suppressor (32.5% of our patients), of which nearly all are characterized (30.7%). This frequency data is similar to that found with the use of tissue biopsy (16). The frequency of *MET* alterations in our population was 6% compared with previous studies where the rate of *MET* alterations varied from 6% to 32%, depending on whether ctDNA or tissue was analyzed, the tumor type, and the number of patients evaluated (17–20). Other common alterations were in *EGFR* (13.0%), *KRAS* (12.5%), *PIK3CA* (9.1%) and *APC* (6.6%) genes (Figure 2A). These frequencies were in the range of frequencies described in other studies (8,9,21).

Nearly 48% of all patients had a potentially actionable alteration. Among the 423 patients with alterations detected, 75.7% had a potentially actionable alteration. The rates of potentially actionable alterations in tissue, as reported in other studies, have varied widely (from about 20% to 93%), probably depending on the panel used, the definition of actionability (14) and the time period of the study (21–24). Of special interest was a patient with advanced gastric cancer who showed amplifications in *EGFR* in her ctDNA, a result that was confirmed by tissue NGS, and attained a rapid partial remission after two months of therapy with an anti-EGFR regimen combining an EGFR antibody and small molecule inhibitor. *EGFR* amplification is uncommon in gastric cancer (being seen in about 2–3% of patients). Preclinical studies have shown that a subset of gastric cancers and *EGFR* amplification respond to anti-EGFR agents (25). Further, combining anti-EGFR antibodies

such as cetuximab with small molecule inhibitors such as erlotinib may augment clinical responsiveness (15).

In this study, 37% of patients did not have any detectable alteration. This was most prominent for brain tumors, where 68% of patients (N = 152 total tested) had no discernible alteration in their ctDNA. Bettgowda et al (6) determined that ctDNA can be detected over 75% of 640 patients with various cancer types, but in less than 50% of primary brain, renal, prostate, or thyroid cancers, suggesting that blood-brain barrier could prevent ctDNA from entering the circulation. On the other hand, it is interesting that 31% of our patients with brain tumors did have a discernible aberration on ctDNA testing. If brain tumors are eliminated from the denominator, 72% of patients (374/518) had at least one discernible ctDNA alteration.

Two hundred forty-one patients (36%) had characterized abnormalities that could conceivably be addressed by at least one FDA-approved drug; an additional 79 patients (11.8%) had at least one alteration potentially druggable via in clinical trial. Overall, 76% of patients with alteration(s) had an aberration potentially actionable either by an experimental agent in clinical trials or by an approved drug. Potential actionability with FDA-approved drugs for characterized alterations was common in lung (55% of patients), breast (49%), head and neck (48%), and gastrointestinal cancers (46%).

For lung cancers, *EGFR* alterations were frequent and actionable. An illustrative patient is shown (Figure 4C). This patient had lung and brain metastases, and a first-generation kinase sensitive *EGFR* alteration in the ctDNA (also confirmed by tissue NGS). The patient showed an excellent response to erlotinib in both the brain and the lung, and, as a result, the planned radiation therapy for the brain was aborted. Because ctDNA requires only a small tube of blood, the time to attaining results is generally shorter than for tissue NGS, and the results may therefore give an early readout for therapy that is important for individuals with rapidly progressing disease or with metastases in crucial areas such as the brain.

Other potential clinical implications of ctDNA relate to understanding mechanisms of resistance to targeted agents. We describe an illustrative patient with colon cancer whose tissue NGS initially showed wild-type *KRAS* and was responding to cetuximab-based therapy. At the time of progression, multiple emerging alterations in *ERBB2*, *BRAF*, *KRAS* and *NRAS* were revealed; these alterations may lead to and predict cetuximab resistance (Figure 4D) (26,27).

For breast cancers, many of the actionable alterations were in the PTEN/PI3K/mTOR axis. Several mTOR inhibitors are being investigated or are approved, including everolimus, which is on-label for breast cancer. For head and neck cancers, characterized *PIK3CA* anomalies were also relatively frequent (about 16% of patients) (28). Notably, most of the alterations in *BRCA2* and *EGFR* in head and neck cancer were of unknown significance (and hence not considered potentially actionable).

Although this study included significantly higher numbers of patients than many previous reports, it has several limitations. Most importantly, the database was de-identified and therefore had minimal clinical annotation. As such, staging as well as treatment outcomes

and comparison with tissue biopsy results were not feasible. However, our previous work has shown that about 85% of our patients sent for ctDNA testing have metastatic/recurrent disease (9). Furthermore, our prior study in lung cancer demonstrated that approximately 28 percent of patients received therapy matching 1 ctDNA alteration(s) (29). This rate may be higher in lung cancer than in other tumors because of the availability of matched therapies. In addition, a previous study testing for ctDNA with the current technology demonstrated that important alterations were matched in blood and tissue in 87% of individuals (30).

In conclusion, we have evaluated 670 patients for ctDNA anomalies and demonstrate that a significant subset has detectable alterations. Perhaps surprisingly, about one-third of patients with brain tumors also have discernible altered ctDNA. Many of the abnormalities are at least theoretically druggable. This is exemplified by our patient with gastric cancer and an *EGFR* amplification, an alteration that occurs in less than three percent of patients with this type of tumor, who achieved a 60% reduction in the size of liver metastases after about five months of anti-*EGFR* treatment (case 1, Figure 4A and 4B). Furthermore, repeat testing showed normalization in amplitude of *EGFR* copy number in blood circulation consistent with tumor response. Of interest in this regard, liquid biopsies are non-invasive, and require only a small amount of blood for testing. In contrast, a tissue biopsy can be painful, and may have complications. Even though it has been already published that *EGFR* ctDNA tests may be useful to select treatment (31,32), our patient with lung cancer and brain metastases (Case 2, Figure 4C) exemplifies well how ctDNA was used prospectively to select therapy and how the patient responded when a rapid readout for genomic tests was needed in a setting of urgent treatment decisions. Serial liquid biopsies may also lend themselves to following therapy response and predicting resistance, and ctDNA testing may provide a technology for evaluating actionable alterations in difficult-to-biopsy tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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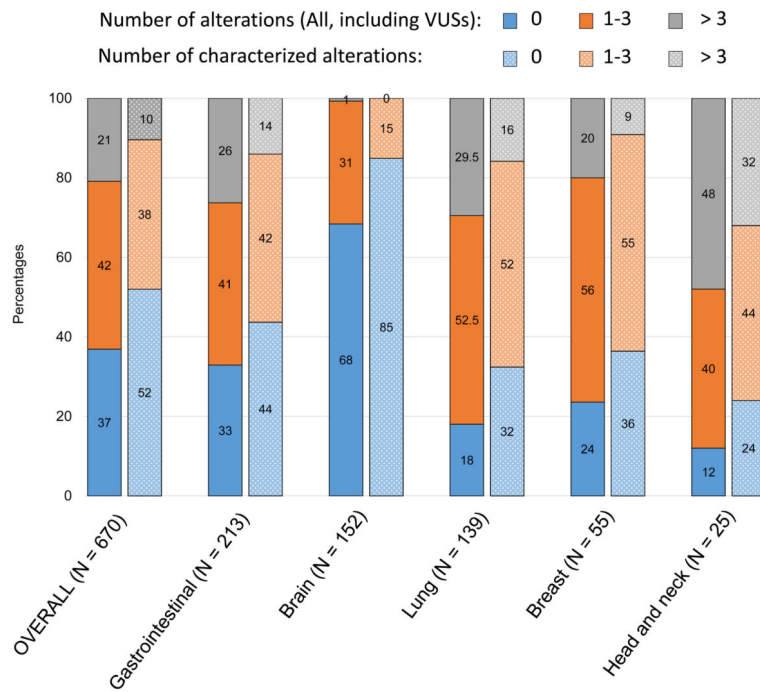


Figure 1. Percentage of patients with a given number of alterations

Bar graph representing the percentage of patients carrying the specified number of alterations, for the most common tumor types (at least 25 patients). For each tumor type, the left bar represents the percentages for all non-synonymous alterations, including variant of unknown significance (VUSs); and the right bar represents the percentages for the characterized alterations only (defined at the gene variant level).

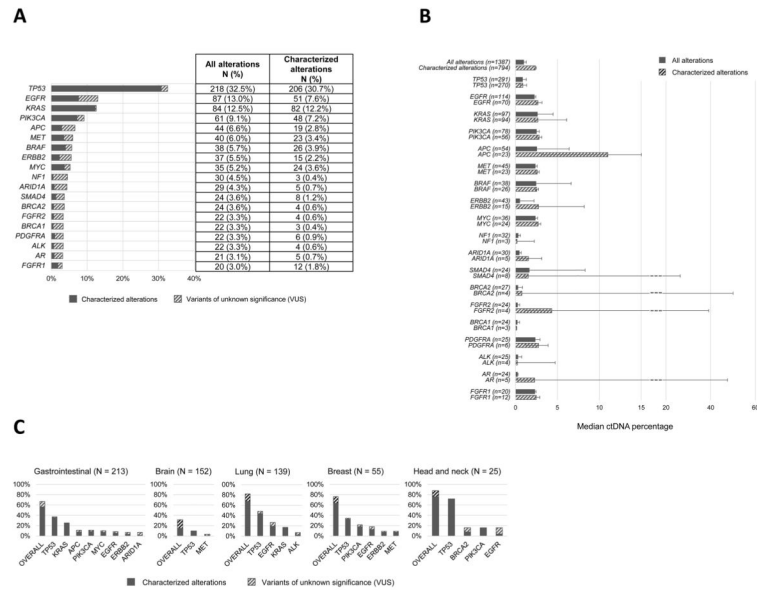


Figure 2. Frequency and quantitation of alterations

A, Frequency of alterations in the overall population (N = 670). Only genes with alterations in 20 patients are displayed; all alterations were non-synonymous. Alterations of unknown significance (variant of unknown significance; VUSs) versus characterized were considered at the variant level. Some patients had multiple alterations in the same gene. Alterations were also identified in the following genes (albeit in less than 3% of patients): *ABL1*, *AKT1*, *ARAF*, *ATM*, *CCND1*, *CCND2*, *CCNE1*, *CDK4*, *CDK6*, *CDKN2A*, *CDKN2B*, *CSF1R*, *CTNNB1*, *ERBB4*, *ESR1*, *EZH2*, *FBXW7*, *FGFR3*, *GATA3*, *GNAQ*, *GNAS*, *HNF1A*, *HRAS*, *IDH1*, *IDH2*, *JAK2*, *JAK3*, *KIT*, *MAP2K1*, *MAP2K2*, *MLH1*, *NFE2L2*, *NOTCH1*, *NRAS*, *NTRK1*, *PROC*, *PTEN*, *RAF1*, *RBI*, *RET*, *RHOA*, *RIT1*, *ROS1*, *SMO*, *SRC*, *STK11*, *TERT*, *TSC1*, and *VHL*.

B, Median ctDNA percentages (95%CI) in patients who had an alteration in the designated gene. Note the break in the scale after 15% of ctDNA to accommodate some of the large 95%CI.

C, Percent of patients that had the designated alteration by tumor type. Bar graph representing the most frequent alterations per tumor type. Percentages given are percent of patients with that tumor type who harbored that alteration. Tumor types with at least 25 patients are represented. Most frequent genes per tumor types are represented (exception for lung tumors: we also included *ALK* because of its potential driver role in the disease, despite the fact that it was only found in 10 patients). All alterations were non-synonymous. The distinction between characterized alterations and variants of unknown significance (VUS) was done at the variant level.

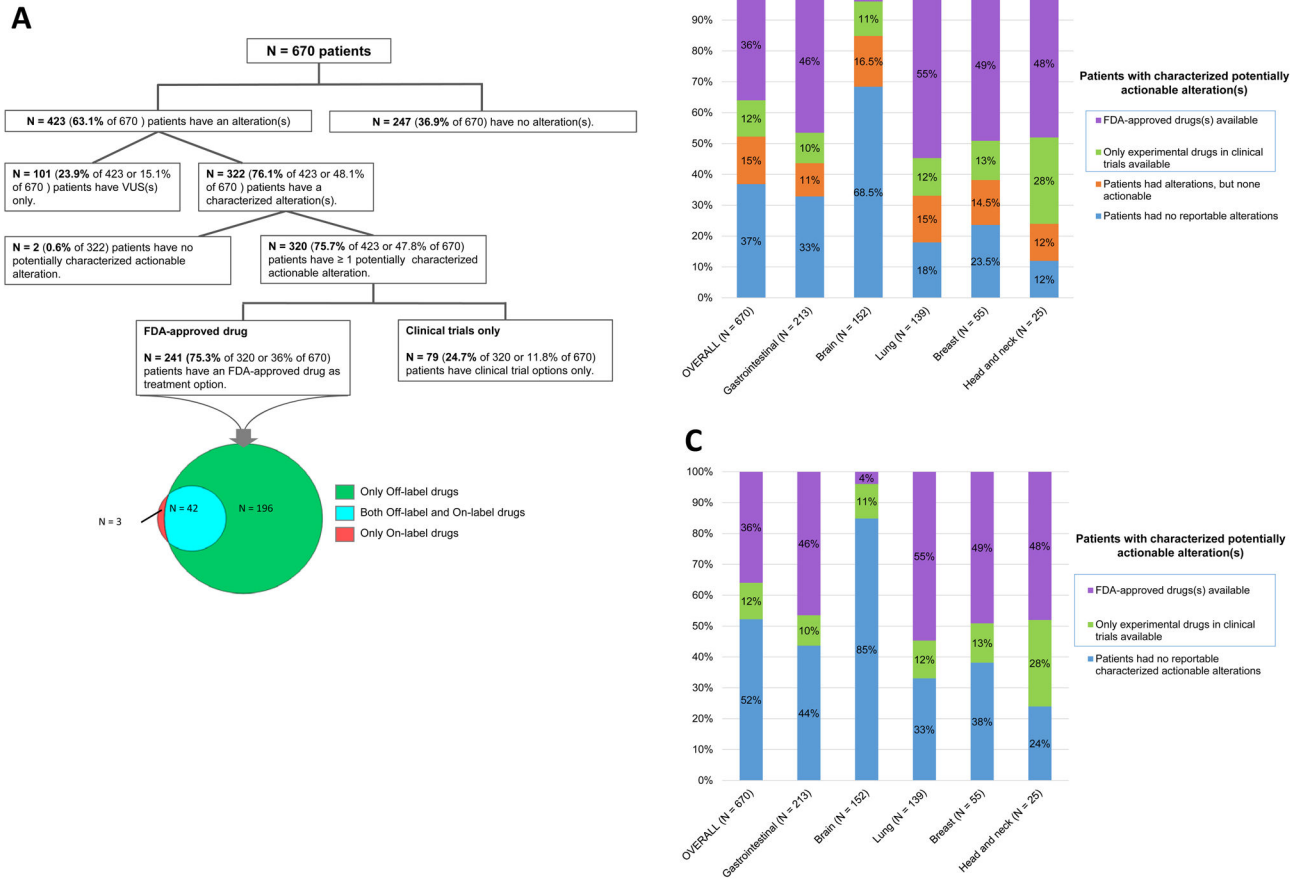


Figure 3. Actionability analysis

A, Actionability diagram in the overall population. Diagram representing the potential actionability of ctDNA alterations in the overall population. In total, 320 patients (47.8% of 670) had at least one theoretically actionable alteration.

B, Potential actionability of alterations by tumor types. Only tumor types with at least 25 patients were represented. * Most of the time, patients were designated as having alterations, but none potentially actionable, because the alterations represented variants of unknown significance (overall 101 of 103 patients): only 2 patients had non-actionable characterized alterations.

C, Potential actionability of characterized alterations by tumor types. Only tumor types with at least 25 patients were represented. * Included patients with no alterations at all (N=247), patients with variants of unknown significance (VUS) only (N=101), or patients with non-actionable characterized alterations (N=2).

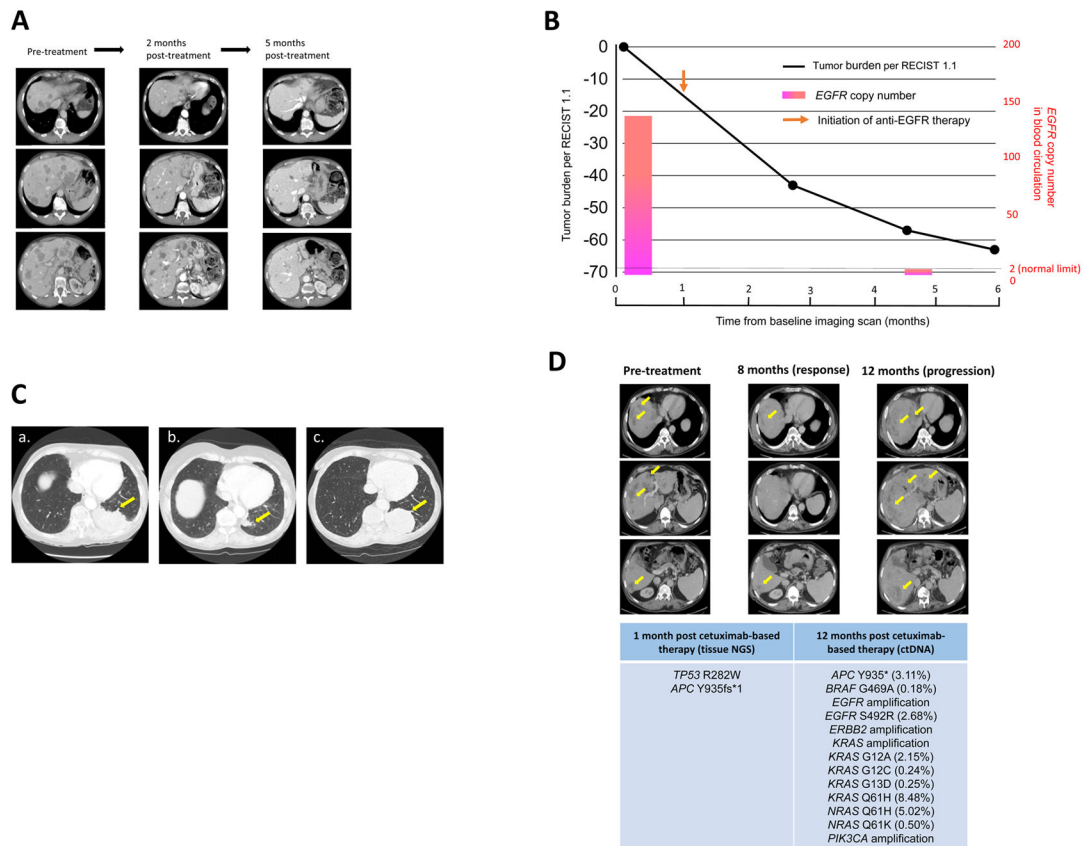


Figure 4. Patient cases

A, Case Study 1: Serial imaging while receiving anti-EGFR therapy. Tissue NGS also showed *EGFR* amplification. Patient received erlotinib and cetuximab demonstrated notable improvement in abdominal fullness and pain after one week. Erlotinib dose was 150 mg PO daily and cetuximab dose was 250 mg/m² intravenously (weekly).

Left, CAT scan of the abdomen before treatment. Middle, CAT scans about two months after treatment shows about 40% reduction in size of liver tumors. Right, CAT scans about five months after treatment showed further reduction of liver tumors (~60 % reduction).

B, Case Study 1: Change in tumor burden and *EGFR* copy number in blood circulation while receiving anti-EGFR therapy. Patient's tumor burden assessed by RECIST 1.1 overall showed 60% tumor reduction five months after the initiation of anti-EGFR therapy. Also observed was a decrease in *EGFR* copy number to reference level (*EGFR* copy number: pre-treatment: 143.9, post-treatment: 2.0 [germline copy number of *EGFR* = 2.0]). Copy number was calculated based on the number of *EGFR* copy molecules in circulation by previously described methods (13).

C, Case Study 2: Serial CT scan images of primary *EGFR*-positive lung mass of a patient with adenocarcinoma of the lung and *EGFR* E746_A750 del alteration in ctDNA treated with anti-EGFR therapy (erlotinib).

Tissue NGS later also showed the *EGFR* alteration. Patient received erlotinib and demonstrated partial response.

a, CAT scan of the chest before treatment.

b, CAT scan after treatment shows significant reduction in tumor size.

c, Eight months after treatment started, patient shows progression. There is no *EGFR* T790M in the ctDNA or tissue and the patient therefore receives chemotherapy.

D, Case Study 3: Patient with *K-RAS* wild-type colon cancer treated with cetuximab-based therapy. ctDNA analysis at progression with multiple resistance alterations.

Patient with *KRAS* wild-type colon cancer was initially responding to the cetuximab-based therapy. Images show, left: pre-treatment, middle: partial response, and right: at progression. At the time of progression, ctDNA was obtained and showed multiple alterations including *EGFR/ERBB2/KRAS/PIK3CA* amplifications, *EGFR* S492R, *BRAF*G469A, *KRAS* G12A/G12C/G13D/Q61H and *NRAS* Q61H alterations that could explain the reason for progression.

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Table 1

Population characteristics

Characteristics	Total patients, N = 670
Gender	
Women	336 (50.1%)
Men	334 (49.9%)
Age (median, range)	62.0 years (5–92)
Turn over time^a (median, 95%CI; range)	15 days (15–16 ; 7–35)
Tumor origin	
Gastrointestinal	213 (31.8%)
Brain	152 (22.7%)
Lung	139 (20.7%)
Breast	55 (8.2%)
Head and neck	25 (3.7%)
Genitourinary	19 (2.8%)
Carcinoma of unknown primary	13 (1.94%)
Skin/Melanoma	10 (1.5%)
Gynecologic	7 (1.0%)
Erdheim-Chester disease	6 (0.9%)
Mesothelioma	5 (0.74%)
Other ^b	26 (3.88%)
Number of patients with 1 alteration (includes characterized alterations and VUSs)	423 (63.1%)
Median number of alterations (range) (includes characterized alterations and VUSs)	1 (0–26)
Number of patients with 1 characterized alteration	322 (48.1%)
Median number of characterized alterations (range) in patients with alterations	1 (0–13)

^aTime from blood collection to results.

^bOther included: Lymphoma, n=4; Neuroendocrine carcinoma, n=4; Sarcoma, n=3; Castleman disease, n=2; Thymoma, n=2; Chordoma, n=1; Desmoid tumor, n=1; Nerve sheath tumor, n=1; Myoepithelial carcinoma, n=1; Thymic carcinoma, n=1; Prolactinoma, n=1; and Unknown diagnosis, n=5.

The majority of lung cancers were adenocarcinomas, with n=118/139 = 84.9% (The 21 remaining cases were squamous cell carcinoma, n=12; small cell carcinoma, n=7; adenosquamous, n=1; carcinoid tumor, n=1)).

Characterized alterations were defined at the gene variant level. VUS: variant of unknown significance.

Table 2

Correlations between patient characteristics and the number of alterations*

All alterations considered (including variants of unknown significance)						
Variables	Univariable			Multivariable**		
	N of alterations median, (95% CI)	P-value	B coef.	t-statistic ^b	P-value	
Overall (N = 670)	1 (1-1)	---	---	---	---	---
Gender						
Women (N = 336)	1 (1-1)	0.318	---	---	---	---
Men (N = 334)	1 (1-1)					
Age at diagnosis^a						
62 years old (N = 349)	1 (0-1)		0.034	1.193		0.233
> 62 years old (N = 321)	2 (1-2)					
Tumor type						
Gastrointestinal vs not (N = 213)	2 (1-2) vs 1 (1-1)		-0.030	-0.815		0.415
Brain vs not (N = 152)	0 (0-0) vs 2 (1-2)		-0.084	-2.387		0.017
Lung vs not (N = 139)	2 (2-3) vs 1 (1-1)		-0.012	-0.326		0.745
Breast vs not (N = 55)	1 (1-2) vs 1 (1-1)		---	---		---
Head and neck vs not (N = 25)	3 (1-6) vs 1 (1-1)		0.069	2.359		0.019
Alterations detected						
<i>TP53</i> vs not (N = 218)	3 (3-4) vs 0 (0-1)		0.268	8.868		< 0.001
<i>EGFR</i> vs not (N = 87)	4 (3-5) vs 1 (1-1)		0.221	7.746		< 0.001
<i>KRAS</i> vs not (N = 84)	4 (3-5) vs 1 (1-1)		0.166	5.494		< 0.001
<i>PIK3CA</i> vs not (N = 61)	5 (4-6) vs 1 (1-1)		0.249	8.682		< 0.001
I gene altered with^c						
<i>cIDNA</i> 10% (N = 101)	5 (4-6)		0.216	7.035		< 0.001
<i>cIDNA</i> < 10% (N = 569)	1 (1-1)					

Characterized alterations considered (excluding variants of unknown significance)

All alterations considered (including variants of unknown significance)					
Variables	N of alterations median, (95% CI)	Univariable		Multivariable**	
		P-value	B coef.	t-statistic ^b	P-value
Overall (N = 670)	0 (0-1)	---	---	---	---
Gender					
Women (N = 336)	0.5 (0-1)	0.619	---	---	---
Men (N = 334)	0 (0-1)				
Age at diagnosis^a					
62 years old (N = 349)	0 (0-0)	0.001	0.015	0.673	0.501
> 62 years old (N = 321)	1 (0.5-1)				
Tumor type					
Gastrointestinal vs not (N = 213)	1 (0-1) vs 0 (0-0)	0.001	-0.004	-0.119	0.905
Brain vs not (N = 152)	0 (0-0) vs 1 (1-1)	<0.001	-0.037	-1.177	0.240
Lung vs not (N = 139)	1 (1-2) vs 0 (0-0)	<0.001	0.018	0.582	0.561
Breast vs not (N = 55)	1 (1-1) vs 0 (0-1)	0.048	-0.003	-0.111	0.911
Head and neck vs not (N = 25)	2 (1-4) vs 0 (0-1)	<0.001	0.064	2.668	0.008
Characterized alterations detected					
<i>TP53</i> vs not (N = 206)	2 (2-2) vs 0 (0-0)	<0.001	0.358	15.163	<0.001
<i>EGFR</i> vs not (N = 51)	3 (2-4) vs 0 (0-0)	<0.001	0.279	12.267	<0.001
<i>KRAS</i> vs not (N = 82)	3 (2-3) vs 0 (0-0)	<0.001	0.241	10.127	<0.001
<i>PIK3CA</i> vs not (N = 48)	4 (3-5) vs 0 (0-0)	<0.001	0.299	13.350	<0.001
I gene altered with^c					
<i>ctDNA</i> 10% (N = 92)	3 (3-4)	<0.001	0.183	7.473	<0.001
<i>ctDNA</i> < 10% (N = 578)	0 (0-0)				

* Only tumor types with > 25 patients were included; only the most prevalent genes were included (genes altered in > 50 patients were included for the upper table and > 40 patients for the lower table).

** Variables with P-values < 0.05 in the univariable analysis were included in the multivariable model.

P-values were calculated using the Mann-Whitney tests for univariable analyses and a linear regression model was computed for the multivariable analysis.

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^aCut-off of 62 years old corresponds to the median (Table 1).

^bThe t-statistic is the ratio of the B coefficient and the Standard Error; the higher the value, the greater is the importance of the variable in the model.

95% CI, 95% Confidence Interval.

^cThe cut-off of 10% corresponds to ten times the median of percentage of ctDNA detected in all the alterations.