Circulating tumor DNA clearance predicts prognosis across treatment regimen in a large real-world longitudinally monitored advanced non-small cell lung cancer cohort

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Abstract

Introduction: Although growth advantage of certain clones would ultimately translate into a clinically visible disease progression, radiological imaging does not reflect clonal evolution at the molecular level. Circulating tumor DNA (ctDNA), validated as a tool for mutation detection in lung cancer, reflects dynamic molecular changes.

Methods: We performed capture-based ultra-deep sequencing on longitudinal plasma samples utilizing a panel consisting of 168 non-small cell lung cancer (NSCLC)-related genes on 949 advanced NSCLC patients with drive mutations to monitor treatment responses and disease progression. Detailed survival analyses regarding the correlations between ctDNA and progression-free survival (PFS)/overall survival (OS) were performed on 248 patients undergoing heterogeneous treatments with a minimum of 2 ctDNA tests.

Results: This study revealed that higher ctDNA abundance ($p=0.012$) and mutation count ($p=8.5\times10^{-4}$) at baseline are associated with inferior OS. The association between ctDNA and PFS/OS was performed in a sub-cohort consisting of 248 patients with a minimum of 2 evaluations beyond baseline. We revealed that patients with ctDNA clearance, not just driver mutation clearance, at any point during the course of treatment are associated with a longer PFS ($p=2.2\times10^{-16}$ HR=0.28) and OS ($p=4.5\times10^{-6}$ HR=0.19) regardless of the type of treatment commenced and the evaluation schedule.

Conclusion: This prospective real-world study demonstrates that ctDNA clearance during treatment can serve as a predictive and a prognostic marker across a wide spectrum of treatment regimens.

Introduction

The treatment of patients with advanced NSCLC has been revolutionized with the development of therapies directed at specific genetic alterations. The characterization of NSCLC into subtypes based on their genetic alterations has significantly improved the therapeutic efficacies of targeted therapies and disease outcomes in a subgroup of patients [1-4]. However, their efficacies are compromised by the development of resistance mechanisms, which inevitably arise in all patients with a median PFS ranging from a few months to a year due to clonal evolution [5-7]. Currently, the response assessment primarily relies on imaging modalities, which do not reflect clonal evolution at the molecular level[8]. Therefore, there is an urgent need to develop improved modalities for monitoring clonal evolution.

The genomic profile of ctDNA, predominantly released by apoptosis and necrosis of cancer cells, has been shown to closely match those of tumor samples [9,10]. It has been validated as surrogate material for mutation detection in NSCLC[11-13]. For instance, plasma and tissue-based genotyping for *EGFR* T790M yielded equivalent clinical outcomes of Osimertinib, supporting plasma genotyping as an alternative diagnostic option[14]. Much effort has been invested in exploring the potential of ctDNA in monitoring responses and assessing the emergence of drug resistance[15-17]. Among patients undergoing epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) treatment, a reduction in the allelic fraction (AF) of EGFR mutation reflects sensitivity to such inhibitors [18]. In addition, ctDNA has been instrumental in revealing novel resistance mechanisms, such as acquired *EGFR* C797S to osimertinib[5], *MET* Y1248H and D1246N to c-Met inhibitors etc [19].

Patients harboring the same mutation exhibit marked differences in treatment responses [2]. Circulating tumor DNA has been proposed to serve as noninvasive real-time biomarker to provide prognostic and predictive information in treatment monitoring [20-22]. The prognostic value of ctDNA has been well-established in detecting minimal residual disease following surgery or treatment with curative intent and is being explored in treatment responses of advanced patients [23-26]. A recent study has shown that the presence of ctDNA at diagnosis and the detection of residual ctDNA at first evaluation were associated with poor prognosis[21]. More work is needed to comprehensively examine its prognostic and predictive values in cohorts consisting of diverse treatment history.

In this prospective real-world study, we performed capture-based ultra-deep targeted sequencing on longitudinal plasma samples to investigate the potential of ctDNA analysis at various treatment milestones in predicting clinical outcomes. We explored the genomic landscape of 1,336 Chinese patients with advanced NSCLC and focused on 248 of them with a minimum of 2 monitoring points to interrogate the predictive and prognostic value of ctDNA as well as to investigate the dynamics of ctDNA upon pharmacological intervention by using a panel consisting of 168 NSCLC-related genes, covering 170KB of human genome.

Materials and Methods

Patient selection

From September 2015 to October 2016, advanced NSCLC (stage IIIB to IV) patients with specified mutations in at least one of the following genes *EGFR*, *ALK*, *ROS 1*, *RET*, *KRAS*, *PIK3CA*, *ERBB2*, *MET*, and *BRAF* were enrolled. Their longitudinal plasma samples were collected at baseline and various points throughout the treatment at multiple participating insititutes. Detailed inclusion criteria were listed in supplemental methods. This study was approved by a central ethic committee at Nanjing General Hospital of Nanjing Command (2016NZKY-003-02). All other centers were covered by this protocol except for First Affiliated Hospital of Guangzhou Medical University (IRB2016-26) and Tianjin Medical School Affiliated General Hospital (IRB2016-050-01). All patients gave informed consent to participate in the study and gave permission for the use of their peripheral blood.

NGS library preparation and Capture-based targeted DNA sequencing

Fragments of size 200–400bp were selected by AMPure beads (Agencourt AMPure XP Kit), followed by hybridization with capture probe baits, hybrid selection with magnetic beads and PCR amplification. Indexed samples were sequenced on Nextseq500 sequencer (Illumina, Inc., USA) with pair-end reads. An average depth of 11,816x was reached.

Statistical Analysis

All statistical tests were conducted in R (version 3.3.1), and all were 2-sided tests unless otherwise specified. For patient characteristics, the differences in distribution of continuous and categorical variables across groups were assessed using Wilcoxon and Fisher exact tests, respectively. Survival tests were conducted using log-rank tests or Cox regression models when a co-variant was included.

Results

Patient demographics and study design

Within the screened population, 949 harbored driver mutation; 245 had no mutation detected and the remaining 142 patients had non-driver mutation. Approximately, 16% patients were treatment-naïve (207/1,336); 71% (949/1,336) were previously treated and the remaining 13.5% (181/1,336) had no treatment history information available. Thirty-one percent of patients (410/1,336) had 1 line of previous treatment; 18.2% (244/1,336) had 2 lines; 11.1% (149/1,336) had 3 lines and the remaining 10.9% (146/1,336) had more than 3 lines of treatment (Figure 1A). The median follow-up time for patients enrolled in our study was 322 days. The median interval for ctDNA analysis was 95 days. Figure 1B depicts detailed treatment history (outer ring) and treatment information during our study (inner ring). Among the 949 patients harbored driver mutations at baseline, 376 patients received matched targeted therapy (MTT) according to sequencing results. A zoom-in view of their treatment prior to and during our study was shown in Supplemental Figure 1. Detailed survival analysis was performed on 248 patients (longitudinal cohort) with 2 or more evaluation time points beyond baseline. A total of 280 patients had 2 or more follow-up tests and 32 of them were excluded due to various reasons. The selection of patients enrolled in the follow-up cohort was depicted in Fiugre1A.

We first compared and contrasted baseline clinical parameters, including gender, age, smoking history, histology, stage, treatment history and metastatic sites between the longitudinal and the screened cohort. Our data demonstrated that the two cohorts were similar in most of parameters, except for gender and the percentage patients with bone metastasis (Supplemental Table 1). The longitudinal cohort had a female predominance,

had more people with bone metastasis and *EGFR* mutations. Since the presence of driver mutations is one of our inclusion criteria and *EGFR* is the most frequently occurred driver mutation in NSCLC, approximately 50% of patients harbored *EGFR* mutations, which are associated with a female predominance. However, such differences do not skew analyses performed in this study.

Landscape of baseline mutation

We performed capture-based ultra deep targeted sequencing on all baseline plasma samples using a panel consisting of 168 genes, spanning 170KB of human genome. The design and validation of this panel has been described by Mao et al [9]. It achieved 95% and 87% by-variant sensitivity in identifying mutations from matched tissue and plasma samples, respectively, excluding copy number variations (CNVs) [9]. DNA obtained from white blood cells (WBCs) was used as a reference to filter out germline mutations. Overall, an average of 11,816x sequencing depth was achieved.

At baseline, we identified 3,503 aberrations spanning 132 genes, including 2,204 single-nucleotide variants (SNVs), 693 insertions or deletions (Indels), 412 copy-number amplifications (CNAs), 80 copy number deletions, and 114 translocations. Approximately, 18% of patients (245/1,336) had no mutations detected from this panel. *EGFR* was the most frequently mutated gene, followed by *TP53*, occurring in 55% and 41% of patients, respectively. Among all genetic aberrations identified, well-established NSCLC driver mutations, including *EGFR*, *KRAS*, *BRAF*, *ERBB2*, *ALK*, *RET* and *ROS1*, comprised 46.9% of all variants identified. The overview of the mutation spectrum is shown in Figure 2A.

Next, we investigated the clinical relevance of baseline max allelic fraction (max AF) and total cell-free DNA (cfDNA). MaxAF was defined as the maximum allelic fraction

among all somatic mutations identified in a plasma sample. Higher maxAF and cfDNA were associated with more advanced M stage, a higher likelihood of bone/liver metastasis and having more organs with lesions (Figure 2B). Interestingly, maxAF showed a more significant correlation than the amount of cfDNA with all clinical features tested.

Overall survival is correlated with baseline ctDNA abundance and mutation load

We performed detailed analysis on the longitudinal cohort to assess the predictive and prognostic potential of ctDNA. First, we investigated the correlation between overall survival (OS) and baseline parameters, including ctDNA abundance and mutation load. Previous studies interrogating the correlation between mutation load at baseline and overall survival have given inconsistent results [27,28]. Our data revealed an inverse correlation between baseline ctDNA amount imputed by the product of maxAF and total amount of cfDNA and OS (p=0.012). The mutation count was also inversely correlated with OS independent of baseline ctDNA amount ($P=8.5x10^{-4}$) (Figure 3A-B). Next, we derived a molecular signature for OS prediction using multi-variate stepwise regression, starting from 6 gens that were individually associated with OS: *CDKN2A*, *EGFR*, *KEAP1*, *KRAS*, *MET* and *POM121L12*. The final molecular signature consists of *KEAP1*, *KRAS* and *MET*. Patients with no mutations in these genes are associated with a longer OS ($p<0.0001$) (Figure 3C).

ctDNA clearance predicts longer progression-free survival and overall survival

In clinical settings, treatment response is typically monitored on a regular interval by radiological imaging, which does not reflect clonal evolution. We interrogated the potential of utilizing ctDNA as a surrogate for monitoring treatment response using our longitudinal cohort, which had at least 2 ctDNA tests. After a median follow-up of 157

days, 166 (66.9%) patients reached disease progression. During the course of treatment, 123 patients, treated with either MTT or chemotherapy, had a minimum of one time of ctDNA clearance, occurring from 1 month to 15 months after the commencement of treatment, with a median PFS of 8.6 months. Fifty patients achieved partial response (PR), 67 achieved stable disease (SD) and 3 achieved progressive disease (PD) as their best response, resulting in an overall response rate (ORR) of 41.7% and a disease control rate (DCR) of 97.5%. Up to June 25, 2017, median overall survival (OS) for this group has not been reached. In contrast, 125 patients with consistent detectable ctDNA throughout the course of treatment had a median PFS of 4.1 months and a median OS of 16.7 months. Among them, 14 achieved PR, 64 achieved SD and 38 achieved PD as their best response, resulting in an ORR of 12.1% and a DCR of 67.2%. Taken together, our data revealed that patients with a minimum of one time ctDNA clearance are associated with a longer PFS ($p=2.2x10^{-16}$; HR=0.28) and OS ($p=4.5x10^{-6}$ HR=0.19) independent of baseline ctDNA amount, regardless of the type of treatment commenced and the time of evaluation (Figure 4A). The baseline clinical parameters including gender, smoking history, stage, treatment history etc of patients with minimum of one time ctDNA clearance and patients with consistent detectable ctDNA were comparable, except for gender (Supplemental Table 2). Furthermore, patients with a minimum of one time ctDNA clearance had a better ORR ($p=3.9x10^{-7}$) and DCR ($p=1.4x10^{-10}$) comparing to patients had detectable ctDNA throughout the course of treatment. The same trend was observed for patients treated with MTT (Figure 4B) but not for patients treated with chemotherapy (Supplemental Figure 2). Circulating tumor DNA clearance can predict PFS (p=0.022) but not OS (p=0.22) in chemotherapy-treated patients after controlling for baseline ctDNA amount. Collectively,

our data demonstrate ctDNA can serve as a valuable real-time biomarker to monitor therapeutic response and its clearance at any point of treatment can predict treatment benefits. This phenomenon reflects clonal response, thus demonstrating the biological nature underlying the clinical response.

Previous studies have reported a reduction in ctDNA amount during treatment is associated with favorable therapeutic efficacy [21]. Next, we evaluated whether ctDNA clearance or certain degree of ctDNA reduction reflected by maxAF would better correlate with treatment efficacies. To derive a binary classifier which can differentiate the population based on treatment efficacy, we conducted a receiving operating curve (ROC) analysis of changes in maxAF during the course of treatment and identified a reduction in maxAF to zero as the optimal cutoff, achieving an area under curve (AUC) of 75% (Figure 4C). Change in maxAF was defined as the ratio of smallest maxAF detected in follow-up evaluations and baseline. Thus, ctDNA clearance but not its reduction upon pharmacological interventions serves as a predictive marker.

Next, we evaluated whether the clearance of driver mutation can provide equal predictive power as ctDNA clearance for PFS. We compared PFS among 3 groups of patients: with all mutation clearance, only driver mutation clearance and with the presence of driver mutations. No difference in PFS was observed for patients with only driver mutation clearance and patients with driver mutation, suggesting only monitoring corresponding driver mutation can not predict PFS (Figure 4D).

Early detection of disease progression

We investigated whether a re-elevation in ctDNA can be detected prior to radiological assessment of disease progression. Due to the heterogeneity of treatments in

our cohort, we focused on patients undergoing osimertinib treatment (n=74) to examine the potential of ctDNA in early detection of PD. Forty patients experienced radiological PD; among whom, 53.1% had at least one liquid biopsy prior to radiological assessment of PD. A comparison of ctDNA level reflected by maxAF among baseline and all points prior to radiological assessment of PD was performed. Fifty-three percent of patients showed an elevation of ctDNA comparing to baseline reflected by either an increase in allelic fraction of any existing mutation or the emergence of new mutation prior to PD assessed by radiological modalities, with an average leading time of 64 days (Figure 5). Collectively, our data demonstrate the ability of ctDNA in reflecting disease progression and can occur prior to radiological PD.

Discussion

Therapeutic response is often assessed using radiological imaging, which does not capture clonal dynamics and evolutionary changes upon pharmacological interventions. Recent studies have demonstrated the potential of ctDNA as a tool for real-time tracking of molecular dynamics to predict treatment response based on residual disease [21,22]. In this study, we evaluated the potential of ctDNA by performing capture-based ultra deep sequencing on longitudinal plasma samples obtained at various treatment milestones from 248 advanced NSCLC patients. Our real world study, comprising both treatment-naïve and previously treated patients, demonstrates ctDNA can serve as a valuable real-time biomarker to monitor therapeutic response and its clearance at any point of treatment can predict treatment benefits. Circulating tumor DNA clearance is defined as no mutation detected from this panel with an average sequencing depth of 11,816x and 0.2% limit of detection. Patients with a minimum of one time ctDNA clearance during the course of treatment were associated with a statistically significant longer PFS and OS. In contrast, patients with detectable ctDNA throughout the course of treatment are associated with inferior treatment response and survival. We cannot rule out the possibility that patients with ctDNA clearance had mutations with AFs below the limit of detection-0.2%.

The prognostic value of ctDNA at first evaluation has been reported in a prospective study comprising only newly diagnosed patients undergoing first-line treatment [21]. Our study, consisting of a heterogeneous population and diverse evaluation schedules, not only confirmed the finding from previous study, but also extended the power of ctDNA analysis in predicting treatment benefits to all patients regardless of treatment history and evaluation time. Furthermore, we demonstrated that ctDNA clearance can translate to clinical benefits

reflected by longer PFS and OS. Our study warrants further investigations to explore the value of ctDNA clearance as surrogate endpoint of efficacy and as a risk stratification factor, differentiating poor and favorable prognosis.

Our study also revealed the potential of ctDNA in early detection of disease progression, preceding imaging modalities with a median lead time of 64 days. This finding echoes with previous studies across a range of different cancer types, including but not limited to breast cancer [29], colorectal cancer and NSCLC[21,30]. However it is important to note this study was not designed to assess how much earlier ctDNA can detect disease progression than imaging modalities; in many patients, ctDNA analysis and CT scans were not performed in close time frame. It is also noteworthy to point out that for heavily treated patients, earlier detection of PD can only offer limited survival benefits due to the exhaustion of treatment options, suggesting the value of early detection of PD at the molecular level in guiding treatment may be better reflected in first-line patients. In most cases, ctDNA analysis was performed after the assessment of PD by imaging modalities. Therefore, further prospective studies are needed to confirm earlier detection of PD by ctDNA and to accurately define the lead time. Prospective studies evaluating the clinical impact of early therapeutic switch based on ctDNA presence instead of imaging modalities are needed to fully validate our findings.

The prognostic and predictive value of ctDNA concentration prior to treatment has always been a controversial issue with conflicting data reported. Most of studies have reported high ctDNA level at baseline is associated with unfavorable PFS and OS; in contrast, others have reported there is no clear correlation [21,31,32]. In this study, we also evaluated the prognostic value of baseline ctDNA amount in relation to OS and revealed

an inverse correlation. The heterogeneity of our cohort, reflected by diverse treatment history, staging and tumor burden, may affect the analysis. Furthermore, a recently published study has shown novel determinants of ctDNA detection in NSCLC, including the degree necrosis, lymph node involvement, lymphovascular invasion, pathological tumor size, Ki67 labelling indices and tumor histology[33]. Therefore, large cohort studies controlling for such factors are needed to accurately define the prognostic and predictive value of baseline ctDNA. Furthermore, we also derived a molecular signature, which predicts OS. There are a few limitations associated with this study, including the heterogeneity of treatment and evaluation time. Because this is a real world study, a few conclusions are limited in scope, such as the lead time of ctDNA in detecting PD comparing to imaging modalities.

To the best of our knowledge, this is the largest real-world study consisting of Chinese NSCLC patients to interrogate the value of ctDNA in monitoring treatment responses. Taken together, our study demonstrated the predictive and prognostic value of ctDNA clearance during treatment in a heterogeneous population with diverse treatment regiments and evaluation schedules. Furthermore, we demonstrated ctDNA analysis coupled with NGS is clinically meaningful in patients with advanced NSCLC by identifying driver mutations and resistance mechanisms to reflect dynamic molecular changes, thus guiding subsequent treatment.

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Figure Legends

Figure 1 Overview of our cohorts. A. Schematic diagram delineates the presence or absence of driver mutations, treatment lines, follow-up time and number of ctDNA performed during the study. We screened 1,336 patients (screened cohort) to arrive at 949 patients with driver mutations to enroll in our study (enrolled cohort). Survival analyses were performed on 248 patients with 2 or more follow-up tests (longitudinal cohort). A total of 280 patients had 2 or more follow-up tests and 32 of them were excluded due to listed reasons. **B**. This diagram illustrates the treatment history and treatment used in our study of the screened cohort. The outer ring represents treatment history and inner ring represents treatment used in our study. Different colors refer to different treatments.

Figure 2 Mutations identified in baseline plasma samples. A. Oncoprint of mutations identified at baseline of the screened cohort. Different colors denote different types of mutations. Top bar represents the number of mutations a patient carries; side bar represents the number of patients carry a certain mutation. Bottom bars provide information regarding histology, gender and treatment history **B.** Clinical characteristics (M stage, presence of bone metastasis, presence of liver metastasis and number of organs with lesions) associated with maxAF and cfDNA. T-test or Pearson correlation test was applied for continuous variables or binary variables, respectively. Boxplots of both variables over the dichotomized clinical features are shown.

Figure 3 Correlation between baseline characteristics and overall survival. A) ctDNA B) mutation count. C) A signature consisting of KEAP1, KRAS and MET can predict OS. Patients with no mutation in the above 3 genes have a longer OS than patients with mutation in any one of the above 3 genes. * denotes p-value derived from cox regression model.

Figure 4 Predictive and prognostic value of ctDNA clearance during the course of treatment. A. Kaplan-Meier curves for PFS and OS in patients with a minimum of one time ctDNA clearance vs patients with consistent detectable ctDNA throughout the course of treatment. **B**. patients treated with MTT. **C**. ROC curve for changes in maxAF during the course of treatment. A reduction of maxAF to zero is the optimal cutoff with an AUC of 75%. D. Kaplan-Meier curves for PFS in patients with driver mutation clearance, all mutation clearance and patients with the presence of both driver and other mutations throughout the course of treatment.

Figure 5 Detection of ctDNA as a function of time in patients treated with osimeritinib. Circulating DNA analysis schedule was depicted for each patient treated with osimertinib. Each line represents a patient. The length of each line corresponds to the duration of monitoring by ctDNA. The color dot at the beginning of each line represents the clinical response. Green represents no molecular progression prior to radiological progression (no re-elevation of ctDNA or emergence of new mutation); Yellow represents molecular progression proceeds radiological PD. Purple represents patients with fast progression. They achieved radiological PD at first evaluation. Grey dots represent patients without enough information to assess the sequence of radiological PD or molecular PD primary due to infrequent testing. X represents ctDNA clearance. Solid red squares represent molecular PD. Empty red squares represent no molecular PD. Solid black dots represent radiological PD; empty black dots represent radiological SD or PR.

References

1. Mok TS, Wu YL, Thongprasert S, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. *The New England journal of medicine.* Sep 03 2009;361(10):947-957.
2. Janne PA, Yang JC, Kim DW, et al. AZD9291 in EGFR inhibitor-resistant non-small-cell lung cancer. *The New England journal of medicine.* Apr 30 2015;372(18):1689-1699.
3. Mazieres J, Zalcman G, Crino L, et al. Crizotinib therapy for advanced lung adenocarcinoma and a ROS1 rearrangement: results from the EUROS1 cohort. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology.* Mar 20 2015;33(9):992-999.
4. Shaw AT, Ou SH, Bang YJ, et al. Crizotinib in ROS1-rearranged non-small-cell lung cancer. *The New England journal of medicine.* Nov 20 2014;371(21):1963-1971.
5. Thress KS, Paweletz CP, Felip E, et al. Acquired EGFR C797S mutation mediates resistance to AZD9291 in non-small cell lung cancer harboring EGFR T790M. *Nat Med.* Jun 2015;21(6):560-562.
6. Engelman JA, Zejnullahu K, Mitsudomi T, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science.* May 18 2007;316(5827):1039-1043.
7. Frampton GM, Ali SM, Rosenzweig M, et al. Activation of MET via diverse exon 14 splicing alterations occurs in multiple tumor types and confers clinical sensitivity to MET inhibitors. *Cancer discovery.* Aug 2015;5(8):850-859.
8. Colombi D, Di Lauro E, Silva M, et al. Non-small cell lung cancer after surgery and chemoradiotherapy: follow-up and response assessment. *Diagnostic and interventional radiology.* Nov-Dec 2013;19(6):447-456.
9. Mao X, Zhang Z, Zheng X, et al. Capture-Based Targeted Ultradeep Sequencing in Paired Tissue and Plasma Samples Demonstrates Differential Subclonal ctDNA-Releasing Capability in Advanced Lung Cancer. *Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer.* Apr 2017;12(4):663-672.
10. Jovelet C, Ileana E, Le Deley MC, et al. Circulating Cell-Free Tumor DNA Analysis of 50 Genes by Next-Generation Sequencing in the Prospective MOSCATO Trial. *Clinical cancer research : an official journal of the American Association for Cancer Research.* Jun 15 2016;22(12):2960-2968.
11. Bettegowda C, Sausen M, Leary RJ, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Science translational medicine.* Feb 19 2014;6(224):224ra224.
12. Spellman PT, Gray JW. Detecting cancer by monitoring circulating tumor DNA. *Nat Med.* May 2014;20(5):474-475.
13. Yong E. Cancer biomarkers: Written in blood. *Nature.* Jul 31 2014;511(7511):524-526.
14. Oxnard GR, Thress KS, Alden RS, et al. Association Between Plasma Genotyping and Outcomes of Treatment With Osimertinib (AZD9291) in Advanced Non-Small-Cell Lung Cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology.* Oct 01 2016;34(28):3375-3382.
15. Siravegna G, Marsoni S, Siena S, Bardelli A. Integrating liquid biopsies into the management of cancer. *Nature reviews. Clinical oncology.* Mar 02 2017.
16. Crowley E, Di Nicolantonio F, Loupakis F, Bardelli A. Liquid biopsy: monitoring cancer-genetics in the blood. *Nature reviews. Clinical oncology.* Aug 2013;10(8):472-484.
17. Alix-Panabieres C, Pantel K. Clinical Applications of Circulating Tumor Cells and Circulating Tumor DNA as Liquid Biopsy. *Cancer discovery.* May 2016;6(5):479-491.

18. Thress KS, Brant R, Carr TH, et al. EGFR mutation detection in ctDNA from NSCLC patient plasma: A cross-platform comparison of leading technologies to support the clinical development of AZD9291. *Lung cancer.* Dec 2015;90(3):509-515.

19. Li AN, Yang J, Zhang XC, et al. Acquired MET Y1248H and D1246N mutations mediate resistance to MET inhibitors in non-small cell lung cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research.* Apr 10 2017.

20. Parkinson CA, Gale D, Piskorz AM, et al. Exploratory Analysis of TP53 Mutations in Circulating Tumour DNA as Biomarkers of Treatment Response for Patients with Relapsed High-Grade Serous Ovarian Carcinoma: A Retrospective Study. *PLoS medicine.* Dec 2016;13(12):e1002198.

21. Pecuchet N, Zonta E, Didelot A, et al. Base-Position Error Rate Analysis of Next-Generation Sequencing Applied to Circulating Tumor DNA in Non-Small Cell Lung Cancer: A Prospective Study. *PLoS medicine.* Dec 2016;13(12):e1002199.

22. Wan JC, Massie C, Garcia-Corbacho J, et al. Liquid biopsies come of age: towards implementation of circulating tumour DNA. *Nature reviews. Cancer.* Apr 2017;17(4):223-238.

23. Klco JM, Miller CA, Griffith M, et al. Association Between Mutation Clearance After Induction Therapy and Outcomes in Acute Myeloid Leukemia. *Jama.* Aug 25 2015;314(8):811-822.

24. Tie J, Wang Y, Tomasetti C, et al. Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II colon cancer. *Science translational medicine.* Jul 06 2016;8(346):346ra392.

25. Reinert T, Scholer LV, Thomsen R, et al. Analysis of circulating tumour DNA to monitor disease burden following colorectal cancer surgery. *Gut.* Apr 2016;65(4):625-634.

26. Beaver JA, Jelovac D, Balukrishna S, et al. Detection of cancer DNA in plasma of patients with early-stage breast cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research.* May 15 2014;20(10):2643-2650.

27. Shi W, Jiang T, Nuciforo P, et al. Pathway level alterations rather than mutations in single genes predict response to HER2-targeted therapies in the neo-ALTTO trial. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO.* Jan 01 2017;28(1):128-135.

28. Rosenberg JE, Hoffman-Censits J, Powles T, et al. Atezolizumab in patients with locally advanced and metastatic urothelial carcinoma who have progressed following treatment with platinum-based chemotherapy: a single-arm, multicentre, phase 2 trial. *Lancet.* May 07 2016;387(10031):1909-1920.

29. Dawson SJ, Tsui DW, Murtaza M, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. *The New England journal of medicine.* Mar 28 2013;368(13):1199-1209.

30. Newman AM, Bratman SV, To J, et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. *Nat Med.* May 2014;20(5):548-554.

31. Pietrasz D, Pecuchet N, Garlan F, et al. Plasma Circulating Tumor DNA in Pancreatic Cancer Patients Is a Prognostic Marker. *Clinical cancer research : an official journal of the American Association for Cancer Research.* Jan 01 2017;23(1):116-123.

32. Bidard FC, Madic J, Mariani P, et al. Detection rate and prognostic value of circulating tumor cells and circulating tumor DNA in metastatic uveal melanoma. *International journal of cancer.* Mar 01 2014;134(5):1207-1213.

33. Abbosh C, Birkbak NJ, Wilson GA, et al. Phylogenetic ctDNA analysis depicts early-stage lung cancer evolution. *Nature.* Apr 26 2017;545(7655):446-451.