Title: Individualized ctDNA Fingerprints to Monitor Treatment Response and Recurrence in Multiple Cancer Types

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One Sentence Summary: ctDNA fingerprint panels were customized to predict the treatment response for multiple cancer types from individual whole-exome sequencing data.

Abstract: Circulating tumor DNA (ctDNA) panels hold high promise of accurately predicting the therapeutic response of tumors while being minimally invasive and cost-efficient. However, their use has been limited to a small number of tumor types and patients. Here, we developed individualized ctDNA fingerprints suitable for most patients with multiple cancer types. The panels were designed based on individual whole-exome sequencing data in 521 Chinese patients and targeting high clonal population clusters of somatic mutations. Together, these patients represent 12 types of cancers and seven different treatments. The customized ctDNA panels have a median somatic mutation number of 19, most of which are patient-specific rather than cancer hotspot mutations; 66.8% of the patients were ctDNA-positive. We further evaluated the ctDNA content fraction (CCF) of the mutations, and analyzed the association

between the change of ctDNA concentration and therapeutic response. We followed up 106 patients for clinical evaluation, demonstrating a significant correlation of changes in ctDNA with clinical outcomes, with a consistency rate of 93.4%. In particular, the median CCF increased by 204.6% in patients with progressive disease, decreased by 82.5% in patients with remission, and was relatively stable in patients with stable disease. Overall, 85% of the patients with a ctDNA-positive status experienced metastasis or relapse long before imaging detection, except for two patients who developed recurrence and metastasis almost simultaneously. The average lead time between the first ctDNA-positive finding and radiological diagnosis was 76 days in three patients that changed from a ctDNA-negative to -positive status. Our individualized ctDNA analysis can effectively monitor the treatment response, metastasis, and recurrence in multiple cancer types in patients with multiple treatment options, therefore offering great clinical applicability for improving personalized treatment in cancer.

[Main Text:]

Introduction

The incidence of cancer is increasing worldwide (1). Despite continuous development of new treatment strategies, the therapeutic effectiveness and recurrence remain difficult to monitor and it is hard to predict disease progress, which is essential for making appropriate clinical decisions. Although radiological imaging and protein biomarker analysis in the blood remain the most common methods of predicting treatment outcome in cancer, they both lack sensitivity and specificity (2, 3). Thus, new methods with high sensitivity and specificity are needed to monitor the treatment response in real-time and assist in the timely adjustment of a therapeutic regimen according to the most recent gene mutation status.

Circulating tumor DNA (ctDNA) has emerged as a new biomarker to assess therapeutic efficacy and detect recurrence. ctDNA is derived from apoptotic or necrotic tumor cells and resides in the blood, allowing for repeatable sampling in a simple and non-invasive manner. Importantly, analysis of ctDNA can provide snapshots of the tumor burden and genomic profile because ctDNA has a short half-life and is highly specific to the tumor from which it is derived (4). Indeed, several studies have demonstrated the value of ctDNA in monitoring the treatment response. In a study of 53 patients with metastatic colorectal cancer (CRC) who received standard first-line chemotherapy, the fold change of the ctDNA level was correlated with the tumor response exhibited by computed tomography (CT) imaging at 8–10 weeks post-treatment (5). In another study, the majority of 46 patients who were initially diagnosed as having non-metastatic triple-negative breast cancer showed metastasis and recurrence after one cycle of neoadjuvant chemotherapy, and all of these metastatic patients were found to be ctDNA-positive (6). In an open phase I clinical trial of the combination of BRAF, EGFR, and MEK inhibitors, patients with the BRAF V600E mutation showed a greater change of ctDNA and a higher ratio of partial response (PR) and complete response (CR) to treatments than the patients with less of a change of ctDNA and showing stable disease (SD) or progressive disease (PD) status. Notably, there was no significant difference in the expression level of the common protein biomarker carcinoembryonic antigen (CEA) between these groups (7). Therefore, ctDNA appears to be

superior to standard radiological and protein tumor markers in predicting the therapeutic response.

In contrast to generic panels containing only tumor hotspot mutations, a ctDNA panel can be tailored to the tumor genetic profile of individual patients. The TRACERx study customized each patient's ctDNA panel based on clonal mutations, which included a median of 11 clonal and six subclonal single-nucleotide variants (SNVs) per patient (8). The TRACERx study demonstrated that the tumor volume correlated with the allele variation frequency of the average clonal SNVs, and that dynamic measurement of plasma ctDNA could predict and confirm tumor recurrence earlier than clinical CT imaging (8). In another study, a ctDNA panel with 9–24 founding mutations detected residual disease in five out of the six patients with breast cancer after adjuvant therapy; the failure in detecting residual disease for the last patient was attributed to an insufficient blood sample for ctDNA analysis (9). These studies highlighted the successful use of customized ctDNA panels in monitoring lung and breast cancers.

Here, we developed a new platform of individualized ctDNA panels designed based on whole-exome sequencing (WES) data of individual patients. We used SciClone tools (10) to calculate and select the top 20–40 somatic mutations within high clonal population clusters to custom individualized ctDNA panels, and the ctDNA content fraction (CCF) was estimated based on the proportion of these acellular tumor somatic mutant DNA fragments in total cell-free DNA. We also used a pre-designed ctDNA panel with eight hotspot cancer genes to monitor acquired resistance mutations for comparison. We applied the new platform to detect ctDNA in longitudinally collected plasma samples from 521 Chinese patients who represented more than 12 tumor types and had received seven types of treatments, and explored the ability of using ctDNA to predict the treatment response and recurrence.

Results

Validation of the individualized ctDNA fingerprint assay in reference and tumor samples

To determine the detection threshold and sensitivity of the individualized ctDNA assay, we performed more than 150 tests for each variant with a gradient of allelic frequencies in the range of 0–15%. Ten nanograms of ctDNA was used for each test. The background ctDNA value in the wild-type reference samples was $0.065\% \pm 0.062\%$ (mean $\pm$ standard deviation). We set the sum of the mean and standard deviation, 0.127%, as the threshold, which represents the upper confidence level of background noise. The specificity of detecting an individual mutation in the wild-type samples was 80.3%, and the sensitivities of reference samples with expected allele frequencies of 0.1%, 0.25%, and 0.5% were 40.6%, 75.0%, and 96.3%, respectively. Mutations with a known frequency greater than 1% were all successfully detected (Supplementary Table S1). Since each allele has two copies, the threshold CCF for positive detection is twice the reference sample threshold at about 0.25%.

In addition to the sensitivity of a single variant, we calculated the sensitivities of a panel containing five and 10 mutants at concentrations of 0.10%, 0.25%, and 0.50% standard references, which were 44.4% and 46.0%, 97.6% and 99.8%, and 99.99% and 100%, respectively. The specificities of the five and 10 mutants in wild-type samples were 98.7% and 99.9% respectively. Therefore, a panel with more tumor-specific SNVs shows higher sensitivity

and specificity. Based on these results, patients with CCF values higher than 0.25% were considered to be ctDNA-positive, although CCF values were still recorded for those lower than 0.25%.

Patients and study design

Among the 552 cancer patients recruited in this study, WES data were obtained from 521 patients who had case-matched tumor specimen and blood samples. Individualized ctDNA assays were designed for each patient based on WES data using a median of 26 pairs of primers, ranging from 7 to 39. After filtering out amplicons with low amplification efficiency and poor quality, the number of amplicons included in the individualized ctDNA fingerprint assays ranged from 4 to 33, with a median of 19. All patients underwent at least two independent ctDNA tests, with some patients undergoing more than three tests. In total, 1436 individualized ctDNA tests were performed on the 521 patients. Among them, 106 patients received follow-up clinical evaluations: 80 patients had one follow-up visit, 21 patients had two follow-up visits, and five patients had three or more follow-up visits. The flow of the study design is illustrated in Figure 1.

Fig. 1. Patient characterization and flow chart of the study design. The dotted red box shows the pre-designed ctDNA panel and individualized ctDNA panel design.

Application of individualized ctDNA fingerprint assays across multiple tumor types

The distribution of tumor types across the 521 Chinese patients is shown in Figure 2A. The highest number of patients had colorectal cancer (CRC), followed by liver cancer, glioma [combined result of blood and cerebrospinal fluid (CSF) tests], and lung cancer in significant numbers; other cancer types included gastric cancer, cholangiocarcinoma, and pancreatic cancer. For the 15 patients with glioma, we additionally collected CSF along with the total 70 plasma samples. Overall, 66.8% of the patients were positive for ctDNA, and 50.9%, 43.0%, and 25.7% of the patients had detectable ctDNA with a CCF above 0.5%, 1%, and 5% respectively. In the glioma samples, ctDNA detection in the blood was significantly lower than that in the CSF (24.3% vs. 80.0%, $p < 0.01$; Figure 2B), presumably due to blockage of the blood-brain barrier (BBB).

Fig. 2. Overview of ctDNA detection. (A) Distribution of tumor types in the 521 patients. (B) Fraction of patients with a ctDNA detection rate above 0.25% (blue), 0.5% (orange), 1% (light green), and 5% (yellow) in different cancer types.

We also tested for acquired drug-related mutations in 16 patients using a pre-designed ctDNA panel with eight hotspot cancer genes (BRAF, EGFR, ERBB2, KIT, KRAS, MET, NRAS, and PIK3CA). The majority of these patients had colon ($n = 6$) and lung ($n = 3$) cancers: all CRC patients acquired a KRAS mutation with a frequency $>0.05\%$; two of the lung adenocarcinoma patients acquired the EGFR p.T790M mutation, one with a frequency of 3.72% and the other 0.48%; and PIK3CA, KIT, NRAS, and KRAS mutations were detected in patients with cholangiocarcinoma, hepatocellular carcinoma, gastrointestinal stromal and germ cell tumors, and breast, pancreatic, and head and neck cancers (Supplementary Table S2).

Association between ctDNA dynamics and clinical outcome

We followed up 106 patients with regards to clinical outcomes. Among them, 26 patients had more than one clinical evaluation, which allowed us to assess the correlation between the changes in ctDNA levels and clinical evaluation at corresponding times. Of the 136 ctDNA datasets, 127 (93.4%) were consistent with the clinical evaluation. The consistency rates reached up to 100% for most of the major tumor types except for intracranial tumors at 71.4% (15/21) and pancreatic cancer at 60.0% (3/5).

For the consistent datasets, although the patients with lung cancer and urinary tract cancer received only two treatments, other patients experienced multiple treatments, including chemotherapy, radiotherapy, chemoradiotherapy, targeted therapy, immunotherapy, and combined chemotherapy and targeted therapy. In addition, some blood samples were taken from patients both just after surgery and during drug withdrawal, defined as postoperation and no treatment, respectively. Most of the surgical patients underwent tumor resection, whereas six received liver transplantation (Table 1). Among these different types of cancers and treatments, the change in ctDNA as determined by the individualized panel was consistent with clinical observations of PD, SD, and remission (Fig. 3A and B). As expected, the consistency rate in the peripheral blood samples was much lower than that in the CSF for glioma patients, at 66.7% and 100%, respectively, reflecting the physical inhibition by the BBB.

Table 1. Distribution of treatment types in different cancer types.

	Postoperation	Chemotherapy	Radiotherapy	chemoradiotherapy	Targeted therapy	Targeted therapy + chemotherapy	Immunotherapy	No treatment	Total
Colorectal carcinoma	4	10	0	0	3	10	2	3	32
Hepatocellular carcinoma	13	3	1	0	9	0	0	1	27
Intracranial tumor	0	10	1	4	0	0	0	0	15
Cholangiocarcinoma	1	3	0	0	3	1	3	1	12
Lung cancer	0	0	0	0	9	0	1	0	10
Breast cancer	0	1	0	1	2	2	0	0	6
Esophageal cancer	0	0	1	0	1	1	2	0	5
Pancreatic cancer	0	1	0	0	1	0	1	0	3
Gastric cancer	0	1	1	0	0	0	1	0	3
Urinary tract cancer	0	2	0	0	1	0	0	0	3
Soft tissue sarcoma	0	1	0	0	1	1	0	0	3
Other	0	3	1	1	3	0	0	0	8
Total	18	35	5	6	33	15	10	5	127

Fig. 3. Distribution of treatment responses [progression-free disease (PD), stable disease (SD), and remission] according to different tumor types (A) and treatments (B).

We then analyzed the corresponding ctDNA levels with clinical outcome metrics, including PD, SD, and remission. The median CCF of patients with PD was 2.21%. There was no difference in the median CCF between SD patients and those who experienced remission, at 0.17% and 0.31%, respectively ($p = 0.144$). However, the CCF of the SD and remission patients was one order of magnitude lower than that of the PD patients, representing a statistically significant difference (both $p < 0.001$) (Fig. 4A).

We also analyzed the percentage change in ctDNA levels between different detection times along with the corresponding time of clinical evaluations. The CCF detected at the time of clinical evaluation significantly differed from the levels detected at the last assessment for the patients with PD and remission, but there was no such difference for the SD patients ($p < 0.0001$, < 0.0001 , and $p = 0.0520$, respectively; Wilcoxon signed-rank test). Overall, 67.2% of SD patients showed ctDNA levels below the threshold at two time points, which was considered as a change to 0 (Fig. 4B). However, there was high variability among PD patients, with a median change of 204.6% in ctDNA (Fig. 4B). The receiver operating characteristic (ROC) curve suggested that the cut-off value for predicting PD was a 15% increase in the ctDNA level (Fig. 4C). The maximum change of ctDNA observed was a ~29,000-fold increase, which occurred in a patient with brain metastases of breast cancer (Fig. 4B). After surgical removal of the brain metastases, the patient's condition was relieved, and the ctDNA level substantially dropped down to 0.29%. However, after 3 months, the ctDNA of this patient again increased to 84.4%, which

corresponded with new metastases to the pectoralis major muscle and the brain metastases also recurred.

Among the 43 PD patients, 23 showed metastasis and recurrence (Table 2). In addition to 10 patients who had metastases and relapses identified prior to the first ctDNA test, 13 patients were with metastasis and recurrence were followed-up. Eight patients were at or above the 0.25% threshold at the first ctDNA test, five patients showed a change in the CCF from below the threshold to above and reaching ctDNA-positive status. In summary, all patients with metastasis and recurrence showed a ctDNA-positive status, which could accurately predict metastasis and recurrence. Except for patients P1822 and P2022 who were tested to be ctDNA-positive and underwent imaging metastasis and recurrence at almost the same time, 85% of the patients (11/13) reached the ctDNA-positive status earlier than the imaging diagnosis. The lead time between the first detection of positivity for ctDNA and radiological metastasis or recurrence in patients P2829, P1782, and P1609 was 33, 68, and 128 days, respectively, with an average of 76 days. Moreover, 82.6% (19/23) of the patients had at least one test with a CCF above 1%. Patient P3079 had a ctDNA concentration of 4.99% before the right hepatectomy, and the postoperative ctDNA concentration decreased by 80%, but still considered to be ctDNA-positive, and then the patient experienced relapse after 2 months.

A

	ctDNA levels at clinical evaluation time		
	PD	SD	Remission
Interquartile range (%)	0.65 to 5.93	0.12 to 0.265	0.165 to 0.61
Median (%)	2.21	0.17	0.305
Mean (%)	7.604	0.6907	1.016

B

	Changes of ctDNA corresponding to clinical evaluation		
	PD	SD	Remission
Interquartile range (%)	69.47 to 784	-2.98 to 0	-93.46 to -65.13
Median (%)	204.6	0	-82.47
Mean (%)	1354	-5.012	-75.87

C

Fig. 4. ctDNA concentration change and its use for progression prediction. (A) Distribution of ctDNA concentration in different treatment responses. CCF values were significantly different between PD and SD, and between PD and remission. The p-values were obtained from the Kruskal-Wallis H test. n, number of patients. (B) Change of ctDNA concentration after treatment corresponding to the clinical evaluation. (C) ROC analysis of the ctDNA concentration change. The analysis suggested a threshold value of 15.1% to predict PD.

Table 2. Surveillance of metastasis and recurrence by the individualized ctDNA fingerprint assay.

Patient ID	Cancer Type	Metastasis/Recurrence	ctDNA detection time corresponding to metastasis/recurrence	CCF (%)						Trend
				1st	2nd	3rd	4th	5th	6th	
P965	Soft tissue sarcoma	Metastasis + recurrence	before 1st	5.69	12.96	19.74
P1273	Lung adenocarcinoma	Metastasis	before 1st	0.22	2.21	2.82	25.2	4.1	3.33
P1472	Liver cancer	Recurrence	3rd	0.25	0.22	0.6	3.7	0.4
P1550	Cholangiocarcinoma	Recurrence	3rd	0.59	0.54	0.81
P1609	Colorectal cancer	Metastasis	4th	0.23	0.2	0.29	1.22	0.23	0.36
P1670	Lung adenocarcinoma	Metastasis	before 1st	0.57	34.09	62.89
P1782	Cholangiocarcinoma	Metastasis + recurrence	between 3rd and 4th	0.09	1.31	2.22	5.25	6.87
P1822	Liver cancer	Metastasis + recurrence	3rd	0.1	0.06	2.26	22.75
P1889	other	Metastasis	2nd	0.25	1.02
P1909	Pancreatic cancer	Recurrence	before 1st	5.87	0.47	7.95
P2004	Kidney cancer	Metastasis	before 1st	0.37	1.87	0.28	4.13
P2022	Liver cancer	Recurrence	2nd	0.05	1.39	20.89
P2036	Head and neck cancer	Metastasis	before 1st	0.23	0.19	1.81
P2138	Liver cancer	Metastasis	3rd	1.56	0.61	0.83	0.6
P2245	Liver cancer	Recurrence	3rd	0.79	1.35	5.27	0.34	4.09
P2339	Pancreatic cancer	Metastasis	3rd	0.43	0.54	0.58
P2565	Colorectal cancer	Metastasis	before 1st	9.26	6.62	8.88
P2704	Breast cancer	Metastasis	before 1st	1.02	0.32	0.28
P2829	Colorectal cancer	Metastasis + recurrence	between 2nd and 3rd	0.1	0.46	0.93
P2959	Colorectal cancer	Metastasis + recurrence	before 1st	1.32	3.8
P3028	Liver cancer	Recurrence	3rd	0.48	0.27	0.65
P3079	Liver cancer	Recurrence	between 2nd and 3rd	4.99	1.14	5.83
P2745	Breast cancer	Metastasis + recurrence	before 1st	2.26	0.29	84.44

The clinical responses of nine patients were inconsistent with the dynamic changes of ctDNA. These included five patients with gliomas, two with pancreatic cancers, one with medulloblastoma, and one with breast cancer. Although all of the patients with glioma showed disease progression, their plasma ctDNA levels fluctuated below the threshold, owing to the low detection rate of ctDNA in plasma. This suggests that it is necessary to monitor ctDNA in the CSF, instead of the blood, for glioblastoma patients. A similar situation occurred in patients with pancreatic cancer, for whom disease progressed but the CCF was determined to be below the 0.25% threshold. The CCF values of the patient with medulloblastoma changed from 0.19% to 0.34% with an increase rate of 79%. Although this change was close to that observed in PD patients, the status of this patient was SD. For breast cancer, the CCF of the first and second tests was 27.7% and 21.3%, respectively. Although the CCF slightly reduced, the absolute value was still very high, which may reflect the PD status of this patient.

Dynamics of individual mutations in the individualized ctDNA fingerprint assay

We selected five patients who had at least three clinical evaluations, and analyzed their disease course in detail through tracking their radiological response and ctDNA dynamics, including the overall CCF and individual mutation changes detected in the personalized ctDNA panels. The five patients had lung adenocarcinoma, cholangiocarcinoma with liver transplant surgery, CRC, hepatocellular carcinoma, and breast cancer, respectively, and their changes in ctDNA were consistent with the clinical evaluations (Fig. 5). Patient A received gefitinib for more than one year, and then acquired the EGFR_p.E746_A750del and EGFR_p.T790M mutations. Before imaging confirmed PD in May of 2018, the fraction EGFR_p.T790M mutation slightly increased and then decreased, whereas the EGFR_p.E746_A750del mutation fraction increased continuously. After treatment with osimertinib, the patient achieved a CR and maintained low ctDNA levels. Moreover, the fluctuations in certain mutations observed in patient D and patient E differed from the overall trend of the panel. For patient D, the ctDNA levels of *DPYD* and *IGSF1* increased significantly in the second detection, and that of *PCSK5* increased in the third test, but the overall ctDNA content of the panel decreased in the second and third detections. Patient E also contained an *APIM2* mutation that was distinct from the overall trend of ctDNA changes.

Fig. 5. Stream graph of five patients showing dynamics of individual mutations as well as the overall trend (inset line plot) as detected by ctDNA. The colors indicate different tumor genes and the specific mutations. (A) Lung adenocarcinoma; (B) cholangiocarcinoma with liver transplant surgery; (C) CRC; (D) hepatocellular carcinoma; and (E) breast cancer. For patient D, a table presents the dynamics of three mutations, which were inconsistent with the overall trend.

Patient individualization of customized ctDNA panels

We designed the individualized ctDNA panels based on the patients' own WES data, and analyzed the specificity of this individualization. A total of 144,158 mutations were detected in 521 patients by WES, including 99,490 mutations that were detected only once, accounting for 69.0% of all mutations. The 99,490 unique mutations were successfully incorporated in at least one individualized ctDNA panel, and 7762 (84.9%) of the mutations were incorporated in only one panel; thus, these panels were highly specific to the individual patients.

To calculate the occurrence of common tumor-associated genes in the individualized ctDNA panel, we defined a tumor-associated gene set using the following method. Based on a previous survey including approximately 90 commercially available clinical gene panels developed by 40 different research institutes and companies, a list was generated containing 2054 genes (11). Using this list, we subsequently screened for 377 genes that occurred five or more times in all commercial panels (Supplementary Table S3). These genes were considered to be tumor-associated genes and potentially clinically actionable. Mutations with frequencies greater than 5% were selected from the WES data and compared to these 377 genes; 114 (21.8%) patients did not contain any of the 377 gene mutations on the list, and 365 (70%) patients had 1–3 of these genes, while 42 (8%) patients had more than three of the genes on the list. Similar results were obtained when comparing these 377 genes with the genes in the individualized ctDNA panels.

Discussion

Although proof-of-concept studies have confirmed the clinical value of ctDNA to predict the treatment response (12), an expert panel from the American Society of Clinical Oncology and the College of American Pathologists concluded that most of the clinical validity and practicability of ctDNA testing reported to date are insufficient (13). Several challenges have to be overcome before ctDNA analysis can be translated to clinical application. Toward this end, we developed a strategy to design patient-specific ctDNA panels with high sensitivity and specificity, which can be applied to patients with multiple types of tumors that received several different treatments.

Our panels achieved a specificity of 99.99% when using 10 SNVs with a detection frequency above 0.25%. The specificity was 99.93% in the reference samples. A median of 19 SNVs were detected in our ctDNA fingerprint assays. The ctDNA detection rate reached up to 67% in all patients, and about 80% in patients with CRC, non-small cell lung cancer (NSCLC), and gastric cancer. In comparison, a targeted deep-sequencing study of more than 70 tumor-associated genes in more than 20,000 patients with solid tumors showed a ctDNA detection rate of 85% overall, and 90% in patients with liver cancer, NSCLC, CRC, and gastric cancer (14). The higher detection rate in that study can be attributed to the fact that all of the included patients had been treated for late-stage cancers. This previous study also showed that the ctDNA detection rate was relatively low in brain cancer patients due to the BBB (14), which is consistent with our present findings.

Previous studies showed that ctDNA could indicate the treatment response and detect recurrence earlier than radiographic imaging in CRC patients that received surgery or chemotherapy (15-17). In a study of 20 patients with metastatic breast cancer, 95% of the patients showed ctDNA fluctuations consistent with imaging-determined PD and SD using a patient-specific ctDNA panel, and 53% of the patients showed an increase in ctDNA that occurred earlier than the imaging-detected PD (18). Monitoring with a panel of 61 hotspot genes revealed that a ctDNA increase was associated with clinical relapse and PD in patients with muscle-invasive bladder cancer (19). These studies generally focused on only one type of cancer or one treatment. In contrast, in the present study, we collected data from patients with more than 12 tumor types and performed ctDNA monitoring on seven different treatment options, including liver transplantation. The ctDNA levels we detected in patients with PD, remission, and SD status were significantly different from each other. At the same time, the correlation between the changes of ctDNA level and clinical evaluation was high at 93%.

Immunotherapy has shown great value in cancer treatment and has been approved by the Food and Drug Administration of the USA for the treatment of a variety of cancers (20, 21). Since it is expected to be applied to a large and growing number of patients, there is a large demand to develop effective methods for monitoring the efficacy of immunotherapy. Monitoring ctDNA changes is a promising approach to begin filling this gap. In a recent study in advanced NSCLC patients treated with nivolumab, 100% of the patients showing an objective response (OR) to immunotherapy had reduced ctDNA concentrations with a median change of 87.8%, while 60% of the non-OR patients had increased ctDNA concentrations (22). In the present study, our individualized ctDNA panels successfully tracked PD, remission, and SD in patients with digestive tract cancers who received treatment with PD-L1 blockers and cell immunotherapy. The consistency rate reached up to 100% (10/10) between the trends of ctDNA

and disease status change (Fig. 3). Thus, this method can effectively monitor multiple cancer types and treatment modes.

The ctDNA panels also have advantages over monitoring only single mutations in predicting disease development. The dynamics of a single mutation representation can be inconsistent with clinical assessments in such cases as well as the overall trend of multiple mutations. In a breast cancer patient whose status changed from SD to PD, the ctDNA content of most mutations increased, but the level of *ZFYVE21*, a known breast cancer marker, continued to decrease (18). Similarly, the *NRAS* level, a known melanoma marker, was reduced by 10-fold in a melanoma patient, but the patient's disease nevertheless progressed (23). These reports are consistent with our findings (Fig. 5D-E). Hence, we monitored multiple patient-specific mutations to avoid this problem.

In addition to monitoring disease status, our ctDNA panels also have potential to predict patient prognosis at an earlier stage than currently possible. The ROC analysis identified a 15.1% increase in ctDNA as the optimal threshold for predicting PD. The corresponding AUC value was 0.997, suggesting a high confidence level (Fig. 4C). Importantly, except for patients with recurrence or metastasis identified before the first ctDNA test, 85% of the patients (11/13) with a ctDNA-positive status at the first ctDNA test or who converted to a ctDNA-positive status during follow-up experienced recurrence or metastasis far before these events were detected by imaging. The remaining patients tested as ctDNA-positive and demonstrated recurrence and metastasis by imaging at almost the same time. The average lead time from the first ctDNA-positive finding to radioactive-confirmed metastasis and recurrence in three patients who reached a ctDNA-positive status during follow-up was 76 days. This finding is similar to a previous study in which 68 patients with advanced bladder cancer were followed-up and ctDNA monitoring showed a median lead time of 107 days (24). Given the relatively small sample size of this study, the clinical advantage of individualized ctDNA panels in predicting metastasis and recurrence need to be further explored on a larger scale in the future.

The cost of using individualized ctDNA panels is expected to be much lower than several previously reported ctDNA panels such as Cancer Personalized Profiling by deep Sequencing (CAPP-Seq), targeted error correction sequencing (TEC-Seq), and a commercially developed ctDNA test kit including more than 50 genes (25-28). All of these panels were designed to target tumor hotspot genes, which account for only a minority of the total ctDNA in the plasma. In cancer patients, somatic cell-free DNA (cfDNA) consists of variants derived from both clonal hematopoiesis and the tumor. Although the common tumor mutations also occur in the genome of white blood cells at high frequency, the majority of cfDNA variants are nevertheless derived from white cells produced in clonal hematopoiesis (29, 30). Therefore, detection based on common tumor mutations is of low efficiency and high cost.

In our present study, we used paired WES data from patients to select variants free of clonal hematopoietic cell interference. The resulting ctDNA panels customized for individual patients displayed high specificity for all patients; 85% of the selected mutations were detected only once, about 30% of the patients did not harbor common hotspot tumor genes, and the remaining 70% patients carried only 1–3 hotspot genes. This detection efficiency was achieved by sequencing a total of about 30 patient-specific mutations per individual, and each of these mutations was harbored in a 100-bp amplicon. Thus, the overall sequencing and analysis involves only about 3-kb sequences per individual. This greatly reduces the cost and time of analysis in comparison to those required for panels needing larger sequencing volumes (31).

Nevertheless, the present individualized ctDNA fingerprints assay still has some limitations. Importantly, it requires prior WES analysis of the tumor tissue before ctDNA monitoring, which is impossible for some patients. Additionally, this assay only uses information of SNVs due to the short sequencing length and leaves out information about indels and longer range changes, including gene fusions/chromosomal rearrangements and copy number variations (CNVs), which are highly valuable diagnosis tools for some tumors (32, 33). Although the present platform mainly detects SNVs, there is relatively less information available on related fusions and CNVs. Furthermore, the present study was limited to patients with metastasis and recurrence, which requires further exploration of the full breadth of clinical benefits of these individualized ctDNA panels.

In conclusion, we developed a new ctDNA platform that could successfully monitor the treatment response, recurrence, and metastasis in a variety of cancer types and for patients receiving multiple treatments. Although further large-scale clinical trials are needed to confirm the utility of these individualized ctDNA panels in clinical settings, the panels offer the important advantages of convenience, accuracy, and efficiency, and show promise for improving personalized treatment decisions in a timely manner.

Material and Methods

Patient samples

Patients were recruited from 20 hospitals in China between May 2016 and December 2018. All patients provided written informed consent to use their genomic and clinical data for research purposes. All procedures involving human participation conformed to the ethical standards of the relevant institutions and/or National Research committees, as well as the 1964 Helsinki Declaration and its subsequent amendments or similar ethical standards.

Tumor tissues and blood samples of 552 Chinese patients diagnosed with cancer were collected. The percentage of tumor cells was assessed by pathologists, and only samples with a tumor content greater than 20% were included in this study. A series of plasma samples were collected at intervals set by the treating physicians. Treatment response and recurrence were evaluated by oncologists based on radiological imaging. ctDNA tests were conducted within the same month of radiological imaging for over 90% of the cases. In the remaining 10% of cases, the time between the ctDNA test and radiological imaging was between 1–2 months. All patients were followed up for a median of 6 months (range 1–16 months).

Reference samples used to evaluate the ctDNA detection threshold and performance were prepared from commercial references including Quantitative Multiplex Formalin Compromised (Mild) Reference Standard (cat# HD798, Horizon Discovery, Cambridge, UK), 1% Multiplex I ctDNA Reference Standard (cat# HD778, Horizon Discovery, Cambridge, UK), and 100% Wildtype (Tru-Q 0) (cat# HD752, Horizon Discovery, Cambridge, UK). The reference standards were mixed in different proportions to make serial reference samples including different variants with a gradient allelic frequency of 15%, 10%, 6%, 3%, 1%, 0.5%, 0.25%, and 0%, respectively. Variant frequencies detected in ctDNA measurements were then used to calculate the sensitivity and specificity of the assay.

DNA extraction

For WES analysis, DNA was extracted from the tumor tissue and blood samples previously frozen using Maxwell® RSC Tissue DNA Kit (cat# AS1610, Promega, Madison, WI, USA) and Maxwell® RSC Blood DNA Kit (cat# AS1400, Promega, Madison, WI, USA) respectively. In addition to fresh tumor tissues, the samples included formalin-fixed, paraffin-embedded (FFPE) tissues, which can damage the quality of DNA; therefore, we used NEBNext FFPE DNA Repair Mix (cat# M6630, New England Biolabs, Ipswich, MA, USA) to reduce this damage. For the ctDNA assay, 10 ml of whole blood was collected and transported using Streck's BCT tubes. cfDNA in the plasma and CSF was then extracted using MagMAX™ Cell-Free DNA Isolation Kit (cat# 29319, ThermoFisher Scientific, Waltham, MA, USA) and stored at -20°C until use.

WES analysis

The whole exons of the tumor tissue and matched white blood cells were sequenced. Library preparation was performed using SureSelect Human All Exon Kit v5 (cat# 5990-9857EN, Agilent Technologies, Santa Clara, CA, USA), sequencing was performed on the Illumina Xten platform, and data analysis was carried out as described previously (34).

ctDNA panels

Given the limitations of multiplex PCR platforms for producing homopolymer-associated indel errors (35, 36), the individualized ctDNA panel was mainly designed to detect somatic SNVs in the peripheral blood. We calculated the clonal clusters for each somatic mutation obtained from WES using SciClone tools, which computationally determines the number and composition of genetic clones across one or multiple samples. The clonal clusters were calculated with the following parameters: tumor purity reviewed by pathologists, tumor copy number alterations (CNA), loss of heterozygosity (LOH) ratio in somatic mutation regions, and somatic variant allele frequencies (VAF). Regions of CNA and LOH were inferred from WES data for each patient using VarScan v2.4.2 tools (37). We selected the top 20–40 somatic mutations within high clonal population clusters, which were applied to Ion AmpliSeq™ Designer for multiplex PCR and amplicon sequencing.

The pre-designed ctDNA panel contains tumor hotspot markers *BRAF*, *EGFR*, *ERBB2*, *KIT*, *KRAS*, *MET*, *NRAS*, and *PIK3CA* to monitor acquired resistance mutations. The information of specific genetic mutations is provided in Supplementary Table S4.

Multiplex PCR and amplicon sequencing

A total of 5–10 ng of cfDNA was used as the template in the multiplex PCR and amplified using KAPA2G Fast Multiplex Mix kit (cat# KK5802, KapaBiosystems, Wilmington, MA, USA) for the individualized ctDNA panel and Ion AmpliSeq™ Library Kit 2.0 (cat# 4475345, ThermoFisher Scientific, Waltham, MA, USA) for the pre-designed panel. Amplified products were ligated to barcoded adapters from Ion Xpress Barcode Adapters 1-16 Kit (cat# 4471250, ThermoFisher Scientific), followed by NGS library amplification using KAPA HiFi HotStart ReadyMixPCR Kit (cat# KK2602, KapaBiosystems) for pre-designed ctDNA panel and Ion AmpliSeq™ Library Kit for individualized ctDNA, respectively. All libraries were sequenced using Ion Torrent next-generation sequencing platforms (Ion S5 Systems, ThermoFisher Scientific).

CCF estimation

After sequencing, high-quality data were retained for analysis according to the following criteria: (1) more than 70% of bases scoring Q20 and above; (2) more than 40% of reads mapped to the designed amplicons; (3) average coverage of amplicons higher than 18,000×; (4) >80% amplicons with a coverage above 1000×. The human reference genome hg19 (Feb.2009 GRCh37/hg19) was used for reads alignment with BWA -0.7.12 (38). Samtools-0.1.18 (39) and bedtools-2.17.0 (40) were used to manipulate alignments, and SNVs were called with VarScan v2.4.2 (37) using the mpileup2snp program. We estimated the ctDNA content fraction in patients according to the following formula: $CCF = [(\sum_{k=1}^k CTR_k / k) \times 2]$, in which CTR refers to the selected somatic mutation allele ratio in the ctDNA test, and k refers to the number of mutations.

Supplementary Materials

Supplementary Table S1. Analytical performance and threshold of the multiplex PCR ctDNA assay for single mutation detection.

ctDNA concentration of standard	0%	0.10%	0.25%	0.50%	1%	3%	6%	10%	15%
Number of replicates	157	160	160	160	157	155	157	157	157
Observed allelic frequency									
Mean	0.06465	0.1221	0.2761	0.5774	1.132	3.38	6.358	9.858	15.26
Std. Deviation	0.06247	0.1126	0.1679	0.2678	0.1966	0.4661	0.7514	0.9003	1.142
Std. Error of Mean	0.004985	0.008899	0.01327	0.02117	0.01569	0.03744	0.05997	0.07186	0.09117
Lower 95% CI of mean	0.0548	0.1045	0.2499	0.5356	1.101	3.306	6.239	9.716	15.08
Upper 95% CI of mean	0.0745	0.1397	0.3023	0.6192	1.163	3.454	6.476	10	15.44
Minimum	0	0	0	0.02	0.66	2.1	4.74	7.66	11.69
25% Percentile	0.01	0.03	0.1225	0.3725	1.01	3.05	5.8	9.29	14.31
Median	0.05	0.07	0.285	0.555	1.11	3.41	6.31	9.76	15.26
75% Percentile	0.105	0.2075	0.4	0.76	1.255	3.67	6.95	10.38	15.93

Maximum	0.23	0.41	0.76	1.43	1.77	4.94	8.2	14.43	18.49
Coefficient of variation	96.62%	92.17%	60.81%	46.39%	17.37%	13.79%	11.82%	9.13%	7.49%
Sensitivity (% , threshold = 0.127%)	NA	40.62%	75.00%	96.25%	100%	100%	100%	100%	100%

NA = not applicable

Supplementary Table S2. Acquired drug-related mutations in 16 patients using a pre-designed ctDNA panel

Patient ID	Tumor Type	New mutation	Mutation frequency
tdP1499	Colorectal cancer	<i>KRAS</i> c.183A>C p.Q61H	0.86%
P2074	Colorectal cancer	<i>KRAS</i> c.34G>T p.G12C	0.60%
P2230	Colorectal cancer	<i>KRAS</i> c.34G>T p.G12C	0.43%
P2800	Colorectal cancer	<i>KRAS</i> c.38G>A p.G13D	0.68%
P2997	Colorectal cancer	<i>KRAS</i> c.37G>T p.G13C	1.75%
P3439	Colorectal cancer	<i>KRAS</i> c.183A>T p.Q61H	1.40%
		<i>NRAS</i> c.182A>T p.Q61L	0.98%
		<i>BRAF</i> c.G1780A p.D594N	0.69%
P3489	Lung adenocarcinoma	<i>EGFR</i> c.2369C>T p.T790M	3.72%
P0220	Lung adenocarcinoma	<i>EGFR</i> c.2369C>T p.T790M	0.48%
P3260	Lung adenocarcinoma	<i>PIK3CA</i> c.1636C>A p.Q546K	2.41%
P2842	Breast cancer	<i>PIK3CA</i> c.G1624A p.E542K	12.35%
P3416	Pancreatic cancer	<i>KRAS</i> c.35G>A p.G12D	3.46%
		<i>NRAS</i> c.181C>A p.Q61K	0.99%
		<i>NRAS</i> c.182A>T p.Q61L	0.95%
P3490	Head and neck cancer	<i>PIK3CA</i> c.3139C>T p.H1047Y	0.51%
P3954	Gastrointestinal stromal tumor	<i>KIT</i> c.2467T>A p.Y823N	2.56%
P4789	Cholangiocarcinoma	<i>PIK3CA</i> c.1634A>G p.E545G	0.82%
P5513	Germ cell tumor	<i>KIT</i> c.2466T>G p.N822K	3.81%
P2393	Hepatocellular carcinoma	<i>NRAS</i> c.35G>A p.G12D	0.32%

Supplementary Table S3. A total of 377 genes that occurred five times or more in a survey of 90 commercially available clinical gene panels developed by 40 different research institutes and companies .

	gene_name	panel_count	gene_description
1	KRAS	39	Kirsten rat sarcoma viral oncogene homolog
2	TP53	39	tumor protein p53
3	KIT	38	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
4	MET	37	MET proto-oncogene, receptor tyrosine kinase
5	NRAS	37	neuroblastoma RAS viral (v-ras) oncogene homolog
6	PTEN	37	phosphatase and tensin homolog
7	RET	37	ret proto-oncogene
8	PIK3CA	36	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
9	CDKN2A	36	cyclin-dependent kinase inhibitor 2A
10	ATM	36	ATM serine/threonine kinase
11	BRAF	36	B-Raf proto-oncogene, serine/threonine kinase
12	ALK	35	anaplastic lymphoma receptor tyrosine kinase
13	CDH1	35	cadherin 1, type 1
14	AKT1	35	v-akt murine thymoma viral oncogene homolog 1
15	APC	34	adenomatous polyposis coli
16	MLH1	34	mutL homolog 1
17	FLT3	34	fms-related tyrosine kinase 3
18	ERBB2	34	erb-b2 receptor tyrosine kinase 2
19	EGFR	34	epidermal growth factor receptor
20	FGFR2	33	fibroblast growth factor receptor 2
21	VHL	33	von Hippel-Lindau tumor suppressor, E3 ubiquitin protein ligase
22	STK11	33	serine/threonine kinase 11
23	PTPN11	33	protein tyrosine phosphatase, non-receptor type 11
24	ABL1	33	ABL proto-oncogene 1, non-receptor tyrosine kinase
25	SMAD4	32	SMAD family member 4
26	PDGFRA	32	platelet-derived growth factor receptor, alpha polypeptide
27	JAK2	32	Janus kinase 2
28	FGFR1	32	fibroblast growth factor receptor 1
29	KDR	32	kinase insert domain receptor
30	HRAS	32	Harvey rat sarcoma viral oncogene homolog
31	JAK3	31	Janus kinase 3
32	IDH2	31	isocitrate dehydrogenase 2 (NADP+), mitochondrial
33	IDH1	31	isocitrate dehydrogenase 1 (NADP+)
34	FGFR3	31	fibroblast growth factor receptor 3

35	NPM1	31	nucleophosmin (nucleolar phosphoprotein B23, numatrin)
36	RB1	31	retinoblastoma 1
37	NOTCH1	31	notch 1
38	CSF1R	30	colony stimulating factor 1 receptor
39	GNAQ	30	guanine nucleotide binding protein (G protein), q polypeptide
40	GNAS	30	GNAS complex locus
41	EZH2	30	enhancer of zeste 2 polycomb repressive complex 2 subunit
42	SMO	30	smoothened, frizzled class receptor
43	FBXW7	29	F-box and WD repeat domain containing 7
44	MPL	29	MPL proto-oncogene, thrombopoietin receptor
45	ERBB4	29	erb-b2 receptor tyrosine kinase 4
46	CTNNB1	29	catenin beta 1
47	GNA11	27	guanine nucleotide binding protein (G protein), alpha 11 (Gq class)
48	SRC	26	SRC proto-oncogene, non-receptor tyrosine kinase
49	SMARCB1	26	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1
50	CDK4	25	cyclin-dependent kinase 4
51	HNF1A	25	HNF1 homeobox A
52	BRCA2	23	breast cancer 2
53	NF1	22	neurofibromin 1
54	MAP2K1	22	mitogen-activated protein kinase kinase 1
55	BRCA1	22	breast cancer 1
56	WT1	22	Wilms tumor 1
57	MSH2	21	mutS homolog 2
58	MYC	21	v-myc avian myelocytomatosis viral oncogene homolog
59	ROS1	21	ROS proto-oncogene 1 , receptor tyrosine kinase
60	PTCH1	20	patched 1
61	MSH6	20	mutS homolog 6
62	CCND1	19	cyclin D1
63	NTRK1	18	neurotrophic tyrosine kinase, receptor, type 1
64	PMS2	18	PMS1 homolog 2, mismatch repair system component
65	DNMT3A	18	DNA (cytosine-5-)-methyltransferase 3 alpha
66	TSC1	18	tuberous sclerosis 1
67	RUNX1	18	runt-related transcription factor 1
68	CBL	18	Cbl proto-oncogene, E3 ubiquitin protein ligase
164	GATA2	18	GATA binding protein 2
69	TET2	17	tet methylcytosine dioxygenase 2
70	DDR2	17	discoidin domain receptor tyrosine kinase 2
71	PALB2	17	partner and localizer of BRCA2
72	ESR1	17	estrogen receptor 1
73	TSC2	17	tuberous sclerosis 2

74	ASXL1	17	additional sex combs like 1, transcriptional regulator
75	CEBPA	17	CCAAT/enhancer binding protein (C/EBP), alpha
165	CHEK2	17	checkpoint kinase 2
166	BRIP1	17	BRCA1 interacting protein C-terminal helicase 1
167	MUTYH	17	mutY DNA glycosylase
76	PDGFRB	16	platelet-derived growth factor receptor, beta polypeptide
77	CDK6	16	cyclin-dependent kinase 6
78	SF3B1	16	splicing factor 3b, subunit 1, 155kDa
79	ERBB3	16	erb-b2 receptor tyrosine kinase 3
80	AR	16	androgen receptor
81	MTOR	16	mechanistic target of rapamycin (serine/threonine kinase)
206	KMT2A	16	lysine (K)-specific methyltransferase 2A
82	BAP1	15	BRCA1 associated protein 1
83	BCL2	15	B-cell CLL/lymphoma 2
84	AKT2	15	v-akt murine thymoma viral oncogene homolog 2
85	GATA1	15	GATA binding protein 1 (globin transcription factor 1)
86	CCNE1	15	cyclin E1
87	MAP2K2	15	mitogen-activated protein kinase kinase 2
88	PIK3R1	15	phosphoinositide-3-kinase regulatory subunit 1
89	ETV6	15	ets variant 6
90	IKZF1	15	IKAROS family zinc finger 1
91	MYCN	14	v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog
92	JAK1	14	Janus kinase 1
93	FGFR4	14	fibroblast growth factor receptor 4
94	MYD88	14	myeloid differentiation primary response 88
95	MEN1	14	menin 1
96	AURKA	14	aurora kinase A
97	RAF1	14	Raf-1 proto-oncogene, serine/threonine kinase
168	PAX5	14	paired box 5
169	KDM6A	14	lysine (K)-specific demethylase 6A
98	NTRK3	13	neurotrophic tyrosine kinase, receptor, type 3
99	NF2	13	neurofibromin 2 (merlin)
100	ATRX	13	alpha thalassemia/mental retardation syndrome X-linked
101	TERT	13	telomerase reverse transcriptase
170	NBN	13	nibrin
171	SDHB	13	succinate dehydrogenase complex subunit B, iron sulfur (Ip)
172	CREBBP	13	CREB binding protein
173	U2AF1	13	U2 small nuclear RNA auxiliary factor 1
174	GATA3	13	GATA binding protein 3
175	SUFU	13	suppressor of fused homolog (Drosophila)

207	BCL6	13	B-cell CLL/lymphoma 6
102	IGF1R	12	insulin like growth factor 1 receptor
103	AKT3	12	v-akt murine thymoma viral oncogene homolog 3
104	NOTCH2	12	notch 2
105	MDM2	12	MDM2 proto-oncogene, E3 ubiquitin protein ligase
176	FLCN	12	folliculin
177	SDHD	12	succinate dehydrogenase complex subunit D, integral membrane protein
178	BMPR1A	12	bone morphogenetic protein receptor type IA
179	FH	12	fumarate hydratase
180	EP300	12	E1A binding protein p300
106	MYCL	11	v-myc avian myelocytomatosis viral oncogene lung carcinoma derived homolog
107	FOXL2	11	forkhead box L2
108	RARA	11	retinoic acid receptor, alpha
109	FLT4	11	fms-related tyrosine kinase 4
110	FLT1	11	fms-related tyrosine kinase 1
111	FANCA	11	Fanconi anemia complementation group A
112	SMARCA4	11	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4
113	AXL	11	AXL receptor tyrosine kinase
114	ARID1A	11	AT-rich interaction domain 1A
115	STAG2	11	stromal antigen 2
181	PRKAR1A	11	protein kinase, cAMP-dependent, regulatory subunit type I alpha
182	SDHC	11	succinate dehydrogenase complex, subunit C, integral membrane protein, 15kDa
183	EPCAM	11	epithelial cell adhesion molecule
184	IL7R	11	interleukin 7 receptor
185	CDKN2B	11	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)
186	FANCC	11	Fanconi anemia complementation group C
187	BLM	11	Bloom syndrome, RecQ helicase-like
188	RAD51C	11	RAD51 paralog C
208	SRSF2	11	serine/arginine-rich splicing factor 2
209	BCOR	11	BCL6 corepressor
210	ERCC2	11	excision repair cross-complementation group 2
211	CIC	11	capicua transcriptional repressor
116	BTK	10	Bruton agammaglobulinemia tyrosine kinase
117	MRE11A	10	MRE11 homolog A, double strand break repair nuclease
118	ETV1	10	ets variant 1
119	PIK3R2	10	phosphoinositide-3-kinase regulatory subunit 2
189	TOP1	10	topoisomerase (DNA) I
190	PHOX2B	10	paired-like homeobox 2b
191	TGFBR2	10	transforming growth factor beta receptor II

192	CDC73	10	cell division cycle 73
193	PBRM1	10	polybromo 1
194	NFE2L2	10	nuclear factor, erythroid 2 like 2
195	MAP2K4	10	mitogen-activated protein kinase kinase 4
196	DICER1	10	dicer 1, ribonuclease type III
212	SETBP1	10	SET binding protein 1
213	ZRSR2	10	zinc finger (CCCH type), RNA binding motif and serine/arginine rich 2
214	PHF6	10	PHD finger protein 6
120	CSF3R	9	colony stimulating factor 3 receptor
121	ERG	9	v-ets avian erythroblastosis virus E26 oncogene homolog
122	MCL1	9	myeloid cell leukemia 1
123	MAPK1	9	mitogen-activated protein kinase 1
124	CARD11	9	caspase recruitment domain family member 11
125	PARP1	9	poly(ADP-ribose) polymerase 1
126	CCND2	9	cyclin D2
127	MDM4	9	MDM4, p53 regulator
215	BCR	9	breakpoint cluster region
216	SDHA	9	succinate dehydrogenase complex subunit A, flavoprotein (Fp)
217	CDK12	9	cyclin-dependent kinase 12
218	BARD1	9	BRCA1 associated RING domain 1
219	FANCG	9	Fanconi anemia complementation group G
220	TSHR	9	thyroid stimulating hormone receptor
221	SETD2	9	SET domain containing 2
222	DAXX	9	death-domain associated protein
223	TNFAIP3	9	TNF alpha induced protein 3
224	PPP2R1A	9	protein phosphatase 2 regulatory subunit A, alpha
225	NKX2-1	9	NK2 homeobox 1
226	MITF	9	microphthalmia-associated transcription factor
227	FANCD2	9	Fanconi anemia complementation group D2
228	RAD50	9	RAD50 homolog, double strand break repair protein
229	ERCC1	9	excision repair cross-complementation group 1
278	ERCC5	9	excision repair cross-complementation group 5
279	KMT2D	9	lysine (K)-specific methyltransferase 2D
128	CHEK1	8	checkpoint kinase 1
129	AURKB	8	aurora kinase B
130	CD274	8	CD274 molecule
131	ETV4	8	ets variant 4
230	XPC	8	xeroderma pigmentosum, complementation group C
231	SMAD2	8	SMAD family member 2
232	FANCF	8	Fanconi anemia complementation group F
233	CDK8	8	cyclin-dependent kinase 8

234	SYK	8	spleen tyrosine kinase
235	CYLD	8	cylindromatosis (turban tumor syndrome)
236	JUN	8	jun proto-oncogene
237	PRDM1	8	PR domain containing 1, with ZNF domain
238	TNFRSF14	8	tumor necrosis factor receptor superfamily, member 14
239	SDHAF2	8	succinate dehydrogenase complex assembly factor 2
240	BCL2L1	8	BCL2-like 1
280	FAS	8	Fas cell surface death receptor
281	TCF3	8	transcription factor 3
282	RAC1	8	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)
283	FOXO1	8	forkhead box O1
284	BIRC3	8	baculoviral IAP repeat containing 3
285	RAD51D	8	RAD51 paralog D
286	RRM1	8	ribonucleotide reductase M1
287	ERCC3	8	excision repair cross-complementation group 3
288	ERCC4	8	excision repair cross-complementation group 4
132	DPYD	7	dihydropyrimidine dehydrogenase
133	RICTOR	7	RPTOR independent companion of MTOR, complex 2
134	MED12	7	mediator complex subunit 12
135	NTRK2	7	neurotrophic tyrosine kinase, receptor, type 2
136	PPARG	7	peroxisome proliferator-activated receptor gamma
137	ATR	7	ATR serine/threonine kinase
138	ARAF	7	A-Raf proto-oncogene, serine/threonine kinase
139	PDCD1	7	programmed cell death 1
199	KMT2C	7	lysine (K)-specific methyltransferase 2C
200	POLE	7	polymerase (DNA directed), epsilon, catalytic subunit
241	XPO1	7	exportin 1
242	ARID2	7	AT-rich interaction domain 2
243	CDKN2C	7	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)
244	FANCE	7	Fanconi anemia complementation group E
245	NSD1	7	nuclear receptor binding SET domain protein 1
246	KEAP1	7	kelch like ECH associated protein 1
247	MYB	7	v-myb avian myeloblastosis viral oncogene homolog
248	EPHA3	7	EPH receptor A3
249	IRF4	7	interferon regulatory factor 4
250	ABL2	7	ABL proto-oncogene 2, non-receptor tyrosine kinase
289	PIK3CB	7	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta
290	PIK3CG	7	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma
291	CBLB	7	Cbl proto-oncogene B, E3 ubiquitin protein ligase
292	CALR	7	calreticulin

293	MAX	7	MYC associated factor X
294	CD79B	7	CD79b molecule
295	SOCS1	7	suppressor of cytokine signaling 1
296	BUB1B	7	BUB1B, mitotic checkpoint serine/threonine kinase
297	DDB2	7	damage-specific DNA binding protein 2
298	LMO1	7	LIM domain only 1
299	RUNX1T1	7	runt-related transcription factor 1; translocated to, 1 (cyclin D-related)
300	AMER1	7	APC membrane recruitment protein 1
301	TFE3	7	transcription factor binding to IGHM enhancer 3
302	PTPRD	7	protein tyrosine phosphatase, receptor type, D
303	PIM1	7	Pim-1 proto-oncogene, serine/threonine kinase
304	EXT2	7	exostosin glycosyltransferase 2
305	EXT1	7	exostosin glycosyltransferase 1
306	CRLF2	7	cytokine receptor-like factor 2
307	KDM5C	7	lysine (K)-specific demethylase 5C
140	RHOA	6	ras homolog family member A
141	TYMS	6	thymidylate synthetase
142	CRKL	6	v-crk avian sarcoma virus CT10 oncogene homolog-like
143	CCND3	6	cyclin D3
144	STAT3	6	signal transducer and activator of transcription 3 (acute-phase response factor)
145	UGT1A1	6	UDP glucuronosyltransferase 1 family, polypeptide A1
197	H3F3A	6	H3 histone, family 3A
205	PMS1	6	PMS1 homolog 1, mismatch repair system component
251	SBDS	6	Shwachman-Bodian-Diamond syndrome
252	IKBKE	6	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon
253	MAP3K1	6	mitogen-activated protein kinase kinase 1, E3 ubiquitin protein ligase
254	TMPRSS2	6	transmembrane protease, serine 2
255	HGF	6	hepatocyte growth factor (hepapoietin A; scatter factor)
308	PIK3CD	6	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit delta
309	NUP98	6	nucleoporin 98kDa
310	XPA	6	xeroderma pigmentosum, complementation group A
311	COL1A1	6	collagen, type I, alpha 1
312	RECQL4	6	RecQ helicase-like 4
313	FOXP1	6	forkhead box P1
314	CD79A	6	CD79a molecule
315	PTGS2	6	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
316	NCOA2	6	nuclear receptor coactivator 2
317	IRS2	6	insulin receptor substrate 2

318	CDK2	6	cyclin-dependent kinase 2
319	BIRC5	6	baculoviral IAP repeat containing 5
320	TOP2A	6	topoisomerase (DNA) II alpha
321	RAD21	6	RAD21 cohesin complex component
322	PIK3C2B	6	phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 beta
323	TMEM127	6	transmembrane protein 127
324	REL	6	v-rel avian reticuloendotheliosis viral oncogene homolog
325	SMC3	6	structural maintenance of chromosomes 3
326	CBFB	6	core-binding factor, beta subunit
327	EPHB1	6	EPH receptor B1
328	SPOP	6	speckle type POZ protein
329	AXIN2	6	axin 2
330	DEK	6	DEK proto-oncogene
331	SMC1A	6	structural maintenance of chromosomes 1A
332	WRN	6	Werner syndrome, RecQ helicase-like
333	SUZ12	6	SUZ12 polycomb repressive complex 2 subunit
334	POLD1	6	polymerase (DNA directed), delta 1, catalytic subunit
335	PML	6	promyelocytic leukemia
336	HSP90AA1	6	heat shock protein 90kDa alpha family class A member 1
146	EWSR1	5	EWS RNA binding protein 1
147	CYP2D6	5	cytochrome P450 family 2 subfamily D member 6
148	VEGFA	5	vascular endothelial growth factor A
149	RPTOR	5	regulatory associated protein of MTOR, complex 1
150	SOX2	5	SRY-box 2
151	ETV5	5	ets variant 5
256	SMAD3	5	SMAD family member 3
257	IGF2R	5	insulin like growth factor 2 receptor
258	PAK3	5	p21 protein (Cdc42/Rac)-activated kinase 3
259	IGF2	5	insulin like growth factor 2
260	BRD4	5	bromodomain containing 4
261	HIF1A	5	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
262	HSP90AB1	5	heat shock protein 90kDa alpha family class B member 1
263	FANCL	5	Fanconi anemia complementation group L
264	CDKN1B	5	cyclin-dependent kinase inhibitor 1B (p27, Kip1)
265	PDGFB	5	platelet-derived growth factor beta polypeptide
337	CKS1B	5	CDC28 protein kinase regulatory subunit 1B
338	TET1	5	tet methylcytosine dioxygenase 1
339	PBX1	5	pre-B-cell leukemia homeobox 1
340	CREB1	5	cAMP responsive element binding protein 1
341	PAX3	5	paired box 3

342	DDIT3	5	DNA damage inducible transcript 3
343	POT1	5	protection of telomeres 1
344	NFKB1	5	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
345	GRIN2A	5	glutamate receptor, ionotropic, N-methyl D-aspartate 2A
346	BCL3	5	B-cell CLL/lymphoma 3
347	KLF6	5	Kruppel-like factor 6
348	FUBP1	5	far upstream element (FUSE) binding protein 1
349	MAP3K7	5	mitogen-activated protein kinase kinase kinase 7
350	NUP214	5	nucleoporin 214kDa
351	SSX1	5	synovial sarcoma, X breakpoint 1
352	BCL10	5	B-cell CLL/lymphoma 10
353	BIRC2	5	baculoviral IAP repeat containing 2
354	CDKN1A	5	cyclin-dependent kinase inhibitor 1A (p21, Cip1)
355	ETS1	5	v-ets avian erythroblastosis virus E26 oncogene homolog 1
356	XRCC2	5	X-ray repair complementing defective repair in Chinese hamster cells 2
357	CUX1	5	cut-like homeobox 1
358	BCORL1	5	BCL6 corepressor-like 1
359	TAL1	5	T-cell acute lymphocytic leukemia 1
360	FLI1	5	Fli-1 proto-oncogene, ETS transcription factor
361	PRKDC	5	protein kinase, DNA-activated, catalytic polypeptide
362	ADGRA2	5	adhesion G protein-coupled receptor A2
363	EPHA7	5	EPH receptor A7
364	EPHA5	5	EPH receptor A5
365	BCL11B	5	B-cell CLL/lymphoma 11B (zinc finger protein)
366	TCF12	5	transcription factor 12
367	RAD51	5	RAD51 recombinase
368	CTNNA1	5	catenin alpha 1
369	NOTCH4	5	notch 4
370	EML4	5	echinoderm microtubule associated protein like 4
371	MAML2	5	mastermind like transcriptional coactivator 2
372	TRRAP	5	transformation/transcription domain-associated protein
373	CDH11	5	cadherin 11, type 2, OB-cadherin (osteoblast)
374	MALT1	5	MALT1 paracaspase
375	LRP1B	5	LDL receptor related protein 1B
376	GLI1	5	GLI family zinc finger 1
377	GPC3	5	glypican 3

Supplementary Table S4. Mutations in the pre-designed ctDNA panel

gene	CDS Mutation	AA Mutation	chrom	start	end
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BRAF	c.1803A>T	p.K601N	7	140453132	140453132
BRAF	c.1801A>G	p.K601E	7	140453134	140453134
BRAF	c.1799_1801delTGA	p.V600_K601>E	7	140453134	140453136
BRAF	c.1799_1800TG>AA	p.V600E	7	140453135	140453136
BRAF	c.1799_1800TG>AT	p.V600D	7	140453135	140453136
BRAF	c.1799T>A	p.V600E	7	140453136	140453136
BRAF	c.1798_1799GT>AA	p.V600K	7	140453136	140453137
BRAF	c.1798_1799GT>AG	p.V600R	7	140453136	140453137
BRAF	c.1799T>C	p.V600A	7	140453136	140453136
BRAF	c.1799T>G	p.V600G	7	140453136	140453136
BRAF	c.1798G>A	p.V600M	7	140453137	140453137
BRAF	c.1790T>G	p.L597R	7	140453145	140453145
BRAF	c.1790T>A	p.L597Q	7	140453145	140453145
BRAF	c.1789_1790CT>TC	p.L597S	7	140453145	140453146
BRAF	c.1789C>G	p.L597V	7	140453146	140453146
BRAF	c.1786G>C	p.G596R	7	140453149	140453149
BRAF	c.1781A>G	p.D594G	7	140453154	140453154
BRAF	c.1780G>A	p.D594N	7	140453155	140453155
BRAF	c.1406G>C	p.G469A	7	140481402	140481402
BRAF	c.1406G>T	p.G469V	7	140481402	140481402
BRAF	c.1406G>A	p.G469E	7	140481402	140481402
BRAF	c.1405G>A	p.G469R	7	140481403	140481403
BRAF	c.1397G>T	p.G466V	7	140481411	140481411
BRAF	c.1397G>A	p.G466E	7	140481411	140481411
EGFR	c.865G>A	p.A289T	7	55221821	55221821
EGFR	c.866C>T	p.A289V	7	55221822	55221822
EGFR	c.1793G>T	p.G598V	7	55233043	55233043
EGFR	c.2125G>A	p.E709K	7	55241677	55241677
EGFR	c.2126A>C	p.E709A	7	55241678	55241678
EGFR	c.2155G>A	p.G719S	7	55241707	55241707
EGFR	c.2155G>T	p.G719C	7	55241707	55241707
EGFR	c.2156G>C	p.G719A	7	55241708	55241708
EGFR	c.2235_2249del15	p.E746_A750delELREA	7	55242465	55242479
EGFR	c.2236_2250del15	p.E746_A750delELREA	7	55242466	55242480
EGFR	c.2237_2255>T	p.E746_S752>V	7	55242467	55242485
EGFR	c.2237_2251del15	p.E746_T751>A	7	55242467	55242481
EGFR	c.2239_2248TTAAGAGAAG>C	p.L747_A750>P	7	55242469	55242478
EGFR	c.2239_2256del18	p.L747_S752delLREATS	7	55242469	55242486
EGFR	c.2239_2251>C	p.L747_T751>P	7	55242469	55242481
EGFR	c.2239_2253del15	p.L747_T751delLREAT	7	55242469	55242483
EGFR	c.2239_2247delTTAAGAGAA	p.L747_E749delLRE	7	55242469	55242477
EGFR	c.2240_2257del18	p.L747_P753>S	7	55242470	55242487
EGFR	c.2240_2254del15	p.L747_T751delLREAT	7	55242470	55242484

EGFR	c.2240T>C	p.L747S	7	55242470	55242470
EGFR	c.2254_2277del24	p.S752_I759delSPKANKEI	7	55242484	55242507
EGFR	c.2290_2291ins12	p.A763_Y764insFQEA	7	55248992	55248993
EGFR	c.2303G>T	p.S768I	7	55249005	55249005
EGFR	c.2307_2308insGCCAGCGTG	p.V769_D770insASV	7	55249009	55249010
EGFR	c.2308_2309insCCAGCGTGG	p.V769_D770insASV	7	55249010	55249011
EGFR	c.2311_2312insGCGTGGACA	p.D770_N771insSVD	7	55249013	55249014
EGFR	c.2319_2320insAACCCCCAC	p.H773_V774insNPH	7	55249021	55249022
EGFR	c.2369C>T	p.T790M	7	55249071	55249071
EGFR	c.2497T>G	p.L833V	7	55259439	55259439
EGFR	c.2504A>T	p.H835L	7	55259446	55259446
EGFR	c.2543C>T	p.P848L	7	55259485	55259485
EGFR	c.2573T>G	p.L858R	7	55259515	55259515
EGFR	c.2582T>A	p.L861Q	7	55259524	55259524
ERBB2	c.929C>T	p.S310F	17	37868208	37868208
ERBB2	c.1963A>G	p.I655V	17	37879588	37879588
ERBB2	c.2033G>A	p.R678Q	17	37879658	37879658
ERBB2	c.2264T>C	p.L755S	17	37880220	37880220
ERBB2	c.2324_2325ins12	p.A775_G776insYVMA	17	37880995	37880996
ERBB2	c.2325_2326ins12	p.A775_G776insYVMA	17	37880996	37880997
ERBB2	c.2524G>A	p.V842I	17	37881332	37881332
KIT	c.1255_1257delGAC	p.D419del	4	55589773	55589775
KIT	c.1509_1510insGCCTAT	p.Y503_F504insAY	4	55592185	55592186
KIT	c.1669_1674delTGGAAG	p.W557_K558del	4	55593603	55593608
KIT	c.1669T>G	p.W557G	4	55593603	55593603
KIT	c.1669T>C	p.W557R	4	55593603	55593603
KIT	c.1669T>A	p.W557R	4	55593603	55593603
KIT	c.1669_1683del15	p.W557_E561del	4	55593603	55593617
KIT	c.1670_1675delGGAAGG	p.W557_V559>F	4	55593604	55593609
KIT	c.1675_1677delGTT	p.V559del	4	55593609	55593611
KIT	c.1676T>A	p.V559D	4	55593610	55593610
KIT	c.1676T>C	p.V559A	4	55593610	55593610
KIT	c.1676T>G	p.V559G	4	55593610	55593610
KIT	c.1678_1680delGTT	p.V560del	4	55593612	55593614
KIT	c.1679T>A	p.V560D	4	55593613	55593613
KIT	c.1679T>G	p.V560G	4	55593613	55593613
KIT	c.1727T>C	p.L576P	4	55593661	55593661
KIT	c.1735_1737delGAT	p.D579del	4	55593669	55593671
KIT	c.1924A>G	p.K642E	4	55594221	55594221
KIT	c.1961T>C	p.V654A	4	55594258	55594258
KIT	c.2009C>T	p.T670I	4	55595519	55595519
KIT	c.2387G>A	p.R796K	4	55599261	55599261
KIT	c.2446G>T	p.D816Y	4	55599320	55599320

KIT	c.2446G>C	p.D816H	4	55599320	55599320
KIT	c.2447A>T	p.D816V	4	55599321	55599321
KIT	c.2466T>G	p.N822K	4	55599340	55599340
KIT	c.2466T>A	p.N822K	4	55599340	55599340
KIT	c.2474T>C	p.V825A	4	55599348	55599348
KIT	c.2485G>C	p.A829P	4	55602664	55602664
KRAS	c.437C>T	p.A146V	12	25378561	25378561
KRAS	c.436G>A	p.A146T	12	25378562	25378562
KRAS	c.351A>T	p.K117N	12	25378647	25378647
KRAS	c.351A>C	p.K117N	12	25378647	25378647
KRAS	c.183A>C	p.Q61H	12	25380275	25380275
KRAS	c.183A>T	p.Q61H	12	25380275	25380275
KRAS	c.182A>T	p.Q61L	12	25380276	25380276
KRAS	c.182A>G	p.Q61R	12	25380276	25380276
KRAS	c.182A>C	p.Q61P	12	25380276	25380276
KRAS	c.181C>A	p.Q61K	12	25380277	25380277
KRAS	c.181C>G	p.Q61E	12	25380277	25380277
KRAS	c.175G>A	p.A59T	12	25380283	25380283
KRAS	c.64C>A	p.Q22K	12	25398255	25398255
KRAS	c.57G>T	p.L19F	12	25398262	25398262
KRAS	c.40G>A	p.V14I	12	25398279	25398279
KRAS	c.38_39GC>AT	p.G13D	12	25398280	25398281
KRAS	c.38G>A	p.G13D	12	25398281	25398281
KRAS	c.38G>C	p.G13A	12	25398281	25398281
KRAS	c.38G>T	p.G13V	12	25398281	25398281
KRAS	c.37G>T	p.G13C	12	25398282	25398282
KRAS	c.37G>A	p.G13S	12	25398282	25398282
KRAS	c.37G>C	p.G13R	12	25398282	25398282
KRAS	c.35G>A	p.G12D	12	25398284	25398284
KRAS	c.35G>T	p.G12V	12	25398284	25398284
KRAS	c.35G>C	p.G12A	12	25398284	25398284
KRAS	c.34_35GG>TT	p.G12F	12	25398284	25398285
KRAS	c.34_35GG>CT	p.G12L	12	25398284	25398285
KRAS	c.34G>T	p.G12C	12	25398285	25398285
KRAS	c.34G>A	p.G12S	12	25398285	25398285
KRAS	c.34G>C	p.G12R	12	25398285	25398285
MET	c.1124A>G	p.N375S	7	116340262	116340262
MET	c.3029C>T	p.T1010I	7	116411990	116411990
MET	MET exon 14 skipping	MET exon 14 skipping	7	116412042	116412042
MET	MET exon 14 skipping	MET exon 14 skipping	7	116412043	116412043
MET	MET exon 14 skipping	MET exon 14 skipping	7	116412044	116412044
MET	MET exon 14 skipping	MET exon 14 skipping	7	116412045	116412045
MET	c.3757T>G	p.Y1253D	7	116423428	116423428

MET	c.3803T>C	p.M1268T	7	116423474	116423474
NRAS	c.183A>T	p.Q61H	1	115256528	115256528
NRAS	c.183A>C	p.Q61H	1	115256528	115256528
NRAS	c.182A>G	p.Q61R	1	115256529	115256529
NRAS	c.182A>T	p.Q61L	1	115256529	115256529
NRAS	c.182A>C	p.Q61P	1	115256529	115256529
NRAS	c.181C>A	p.Q61K	1	115256530	115256530
NRAS	c.181C>G	p.Q61E	1	115256530	115256530
NRAS	c.52G>A	p.A18T	1	115258730	115258730
NRAS	c.38G>A	p.G13D	1	115258744	115258744
NRAS	c.38G>T	p.G13V	1	115258744	115258744
NRAS	c.38G>C	p.G13A	1	115258744	115258744
NRAS	c.37G>C	p.G13R	1	115258745	115258745
NRAS	c.37G>T	p.G13C	1	115258745	115258745
NRAS	c.35G>A	p.G12D	1	115258747	115258747
NRAS	c.35G>T	p.G12V	1	115258747	115258747
NRAS	c.35G>C	p.G12A	1	115258747	115258747
NRAS	c.34G>A	p.G12S	1	115258748	115258748
NRAS	c.34G>T	p.G12C	1	115258748	115258748
NRAS	c.34G>C	p.G12R	1	115258748	115258748
PIK3CA	c.G241A	p.E81K	3	178916854	178916854
PIK3CA	c.263G>A	p.R88Q	3	178916876	178916876
PIK3CA	c.C277T	p.R93W	3	178916890	178916890
PIK3CA	c.G317T	p.G106V	3	178916930	178916930
PIK3CA	c.G323A	p.R108H	3	178916936	178916936
PIK3CA	c.A331G	p.K111E	3	178916944	178916944
PIK3CA	c.G353A	p.G118D	3	178917478	178917478
PIK3CA	c.1035T>A	p.N345K	3	178921553	178921553
PIK3CA	c.1258T>C	p.C420R	3	178927980	178927980
PIK3CA	c.1616C>G	p.P539R	3	178936074	178936074
PIK3CA	c.1624G>A	p.E542K	3	178936082	178936082
PIK3CA	c.1633G>A	p.E545K	3	178936091	178936091
PIK3CA	c.1633G>C	p.E545Q	3	178936091	178936091
PIK3CA	c.1634A>C	p.E545A	3	178936092	178936092
PIK3CA	c.1634A>G	p.E545G	3	178936092	178936092
PIK3CA	c.1635G>T	p.E545D	3	178936093	178936093
PIK3CA	c.1635G>C	p.E545D	3	178936093	178936093
PIK3CA	c.1636C>A	p.Q546K	3	178936094	178936094
PIK3CA	c.1636C>G	p.Q546E	3	178936094	178936094
PIK3CA	c.1637A>G	p.Q546R	3	178936095	178936095
PIK3CA	c.1637A>C	p.Q546P	3	178936095	178936095
PIK3CA	c.G1639A	p.E547K	3	178936097	178936097
PIK3CA	c.3062A>G	p.Y1021C	3	178952007	178952007

PIK3CA	c.3073A>G	p.T1025A	3	178952018	178952018
PIK3CA	c.3127A>G	p.M1043V	3	178952072	178952072
PIK3CA	c.3129G>T	p.M1043I	3	178952074	178952074
PIK3CA	c.G3129A	p.M1043I	3	178952074	178952074
PIK3CA	c.T3132A	p.N1044K	3	178952077	178952077
PIK3CA	c.3139C>T	p.H1047Y	3	178952084	178952084
PIK3CA	c.3140A>G	p.H1047R	3	178952085	178952085
PIK3CA	c.3140A>T	p.H1047L	3	178952085	178952085
PIK3CA	c.3145G>C	p.G1049R	3	178952090	178952090
PIK3CA	c.3145G>A	p.G1049S	3	178952090	178952090

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