Original Article

Circulating tumor DNA detection in advanced non-small cell lung cancer patients

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Background: Circulating tumor DNA (ctDNA) is of great value in characterizing gene mutations of non-small cell lung cancer (NSCLC) patients. However, the limited detection loci and high background errors of existing methods limit their applications. Therefore, novel methods are urgently needed for ctDNA mutation detection. In this study, we evaluated the potential of tag sequencing in characterizing the gene mutations of tumors. This is an amplicon-based method combined with molecular barcoding to reduce background errors.

Methods: We performed the tag sequencing technique to screen for NSCLC related gene mutations in plasma ctDNA and matched tissue DNA samples from 20 Chinese advanced NSCLC patients and the reference standard cell free DNA (cfDNA) set with four concentrations to assess its performance.

Results: The overall concordance of epidermal growth factor receptor (*EGFR*) mutations between tissue DNA and plasma ctDNA was 80%, and the sensitivity of *EGFR* mutations detected in plasma ctDNA was 67%. Moreover, our assay could accurately detect (100%) all of the six detected mutations in four concentrations of the reference standard cfDNA and their frequencies were all close to their actual frequencies.

Conclusions: These findings indicated that the tag sequencing method can effectively and stably detect gene mutations in ctDNA, and is suitable for clinical application. Tag sequencing accurately detected the ctDNA mutations, thereby highlighting the application of ctDNA in molecular diagnostics, prognosis prediction and targeted drug selection.

Keywords: Circulating tumor DNA (ctDNA); epidermal growth factor receptor (*EGFR*); non-small cell lung cancer (NSCLC); personalized therapy; tag sequencing

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Introduction

Non-small cell lung cancer (NSCLC) accounts for 85–90% of lung cancers (1). It has been the most common malignancy and the leading cause of cancer death for decades. Currently, the 5-year overall survival for NSCLC is still lower than 15% worldwide (2). Thus precise detection of gene mutations at an early stage is of great benefit to improve patient treatment. Although the tumor tissue biopsies are the gold standard for mutation detection, some inherent shortcomings still exist in clinical practice, such as the tumor heterogeneity (3,4). Therefore, novel detection methods are urgently needed to characterize circulating tumor DNA (ctDNA) mutations.

ctDNA is a class of DNA fragments that originate from tumor cells; it can be extracted from blood plasma (5,6). Due to its sources, ctDNA carries tumor-specific mutations from tumor cells (7). The detection of ctDNA mutations can reflect the overall genetic profiles of tumors, which may avoid the tumor heterogeneity problems in tissue biopsies. Moreover, the ctDNA samples can be obtained easily and repeatedly taken over the process of patient treatment, and thus can be applied to real-time and dynamical monitoring of evolving gene mutations to guide patient treatment (8,9). Therefore, ctDNA is of great value in reflecting the gene mutations of NSCLC patients, thereby highlighting the potential of ctDNA detection in personalized medicine.

In recent years, various methods have been developed to detect ctDNA mutations at different scales, such as polymerase chain reaction (PCR)-based and NGS-based methods (10-13). PCR-based methods can accurately detect ultra-low frequency mutations, but few gene mutations can be detected simultaneously (14). Because cancer development involves multiple gene alterations, it is difficult to comprehensively characterize cancer genetic information with PCR-based methods. The NGS-based methods mainly involve two methods, such as the amplicon-based method and the hybrid capture method (12,13). The high cost of the probes, high background noise, and the longtime of hybridization time limit the application of the hybrid capture method in clinical practice (15). High background noise limits the application of the amplicon-based method. Recently, the amplicon-based method has been combined with molecular barcoding to reduce the background errors (14). This method is called tag sequencing. However, evaluations of its performance are lacing, so its performance needs to be further validated.

In the present study, we evaluated the performance of tag sequencing. We first detected mutations in a reference standard cell free DNA (cfDNA) set with four concentrations (0.0%, 0.1%, 1.0% and 5.0%) to evaluate its performance. Moreover, we analyzed the mutation concordance between plasma DNA and matched tissue DNA by comparing the epidermal growth factor receptor (*EGFR*) mutations in 20 Chinese advanced NSCLC patients.

Methods

Patient features

Twenty NSCLC patients were recruited from Zhujiang Hospital, Southern Medical University, Guangdong Province, China. The Ethical and Protocol Review Committee of Zhujiang Hospital approved the study protocol. Written informed consent was obtained from all patients. *Table S1* lists the detailed clinical information and demographic features of patients.

DNA preparation

For the reference standard cfDNA, a multiplex I cfDNA Reference Standard Set was obtained from Horizon (Horizon Discovery, Cambridge, United Kingdom) with four different concentrations (0.0%, 0.1%, 1.0%, and 5.0%). The multiplex I cfDNA Reference Standard Set included six known mutations, which could be detected by our assay. The allele frequency of each mutation was validated by droplet digital PCR, as shown in *Figure 1*. For the 20 NSCLC patients, plasma and formalin-fixed paraffin embedded (FFPE) tissue samples were examined for mutation profiles. Genomic DNA was extracted from sections of FFPE tissue samples using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Venous blood samples were collected in K2-EDTA tubes. Then the tubes were spun twice, first at 1,800 g for 10 minutes and then at 16,000 g for 15 minutes. The plasma-depleted whole blood was stored at –80 ℃ for cfDNA isolation. ctDNA was extracted from blood using the MagMAX cfDNA extraction kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. From each phlebotomy specimen, 1.5–4 mL (median 2.5 mL) of plasma was profiled to target ~20 ng of cfDNA input into Tag-Seq library preparation (*Table S1*).

NGS library construction

The Oncomine™ Lung cfDNA Kit (Life Technologies)

	Gene	Mutation	WT (%)	0.1% (%)	1% (%)	5% (%)
Expected frequency	*PIK3CA*	p.E545K	0	0.13	1.3	6.3
	KRAS	p.G12D	0	0.13	1.3	6.3
	EGFR	p.V769_D770insASV	0	0.10	1.0	5.0
	EGFR	p.L858R	0	0.10	1.0	5.0
	EGFR	p.T790M	0	0.10	1.0	5.0
	EGFR	p.E746_A750delELREA	0	0.10	1.0	5.0
Detected frequency	*PIK3CA*	p.E545K	0	0.17	0.91	5.55
	KRAS	p.G12D	0	0.28	1.51	5.91
	EGFR	p.V769_D770insASV	0	0.17	0.7	2.83
	EGFR	p.L858R	0	0.17	1.46	2.92
	EGFR	p.T790M	0	0.11	0.71	3.58
	EGFR	p.E746_A750delELREA	0	0.13	1.01	4.25
	TP53	p.A159V	0.12			
	EGFR	p.E17D				0.22
	KRAS	p.Q61R				0.11

p.E545K
p.G12D
p.V769_D770insASV
p.L858R
p.T790M
p.E746_A750delELREA

Figure 1 Frequency of mutations in the reference standard cfDNA. WT, wild type; cfDNA, cell free DNA.

was applied to construct the adapter-ligated library. Library quality control was performed using the Qubit®2.0 and 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). A multiplexed of 16 libraries consisting of 100 pM prepared library per sample, which were then amplified by emulsion PCR on Ion Sphere™ Particles (ISPs) with the IonOneTouch™ 2 Instrument (Life Technologies). Finally, the template-positive ISPs were enriched and loaded onto and run on Ion Proton (Life Technologies). A panel covering more than 150 hotspot mutations in 11 cancer-related genes, such as *EGFR*, *ALK*, *BRAF*, *KRAS*, *PIK3CA*, and *TP53*, was used in this study.

Mutation identification

The adaptor sequences of the raw data were removed, and then the clean reads were mapped to the human reference genome (hg19). The hotspot and targeted region, together with the parameter files associated with the Oncomine™ Lung cfDNA Assay, were loaded into the variantCaller plugin (Life Technologies) to call and to filter the mutations. The mutations were further filtered using the following filters: (I) the minimum allele frequencies ≥0.1%; (II) the minimum coverage of the mutations ≥1,000×. All identified mutations were visually confirmed by using the Integrative Genomics Viewer (IGV) (16).

Amplification refractory mutation system (ARMS)-PCR validation

To validate the mutations detected by tag sequencing in the plasma ctDNA, the matched tissues of 20 NSCLC patients were screened for 29 known *EGFR* mutations (*Table S2*) using ARMS-PCR as previously described (17).

Results

Patient features

Tissues and matched blood samples were obtained from 20 NSCLC patients, including 7 females and 13 males. *Table 1* lists the patient clinical characteristics. Participants in this study cohort were diagnosed with stage IIIB to IV NSCLC, and all had adenocarcinoma (100%). The majority of patients (12/20, 60%) were non-smokers and most of them (18/20, 90%) were treatment naive. In the two treatment-experienced patients, one had received first-line target therapy for the past 1.5 years and the other was undergoing chemotherapy.

NGS data coverage analysis

All samples, consisting of 20 ctDNA samples from NSCLC patients and 4 different concentrations of reference standard

Table 1 Detailed clinical information about 20 NSCLC patients

Characters	Number
Age (years)	
Mean (SD)	63.9 (7.5)
Median [range]	62 [5–80]
Gender, n [%]	
Male	13 [65]
Female	7 [35]
Pathological diagnosis, n [%]	
Adenocarcinoma	20 [100]
Stage, n [%]	
IIIB	5 [25]
IV	15 [75]
Smoking history, n [%]	
Smoker	8 [40]
Non-smoker	12 [60]
Treatment history, n [%]	
Treatment naïve patients	18 [90]
Treatment experienced patients	2 [10]

SD, standard deviation; NSCLC, non-small cell lung cancer.

cfDNA, were sequenced using Ion Proton. The mean depth of NSCLC patient samples was 335,801, and the range was from 111,359 to 489,020. The mean depth of the reference standard cfDNA samples was 122,991, with a range from 52,994 to 178,002.

NGS assay performance evaluated using the cfDNA reference standard set

Evaluation of assay performance, including accuracy and stability, is our research priority. Therefore, we assessed assay performance by sequencing the cfDNA Reference Standard Set with four different concentrations (0.0%, 0.1%, 1.0%, and 5.0%) and then comparing their detected frequencies with their expected frequencies. In the 0.1%, 1.0%, and 5.0% groups, the assay could detect all six mutations and their allele frequencies were close to their expected frequencies (*Figure 1*). The true positive rate of these three groups was 100% and the false positive rate were 0%. In the 0.0% group, none of these six mutations were detected by the assay, which was concordant with their expected frequencies (0%) (*Figure 1*). In this group, the true negative rate was 100% and the false negative rate was 0%. In addition to these six mutations, one more TP53 mutation was detected in the 0.0% group and two more mutations were detected in the 5.0% group, which may be inherent mutations of the engineering cell line (*Figure 1*).

To further evaluate the assay stability, five more repeated experiments were sequenced in the 1% concentration group. In these six repeated experiments, the mean frequencies of four *EGFR* mutations (p.T790M, p.L858R, p.V769_D770insASV and p.E746_A750delELREA) were 0.81%, 0.93%, 0.81% and 1.1%, respectively (*Table S3*). The frequencies of these four mutations were all close to their expected frequencies 1% (*Table S3*). The mean frequencies of *PIKCA3* (p.E545K) and *KRAS* (p.G12D) were 1.21% and 1.32%, respectively. The frequencies of these two mutations were all close to their expected frequencies 1.3% (*Table S3*). The standard deviation (SD) of these six mutations were all less than 0.3%, which may indicate the favorable stability of our assay (*Table S3*).

EGFR mutation concordance in matched tissue DNA and plasma ctDNA

By using our assay, single-nucleotide variants (SNVs) and insertion-deletion polymorphisms (Indels) of the NSCLC-related genes, including *EGFR*, *ALK*, *PIK3CA*, *BRAF*, *KRAS*, *NRAS*, *TP53*, *MAP2K1* and *ERBB2*, were detected in the plasma ctDNA samples of 20 NSCLC patients. *Figure 2* presents the detected mutations for each patient and their frequencies.

Because one of our major objectives was to compare the detection concordance between the matched tissue DNA and plasma ctDNA, we compared the *EGFR* mutations in the plasma and matched tissue samples of 20 NSCLC patients. In the plasma ctDNA, six EGFR mutations were detected: p.T790M, p.L858R, p.E746_A750delELREA, p.G719C, p.G719S, and p.G719A (*Figure 3*). Of the 12 EGFRm+ patients, all were concordant for the common sensitive mutations p.L858R, p.T790M, and p.E746_A750delELREA, however, the plasma test appears to result in false positive G719X calls in 4 cases. The 8 EGFR wild type (wt) patients were all concordant in that no mutations were detected in tissue or plasma samples. The sensitivity and specificity has been shown in *Table S4*. According to patients, the sensitivity and specificity of detection mutations in plasma ctDNA and tissue DNA were 67% and 100%, respectively (*Figure 4*). The accuracy and the concordance were 80% and 80%, respectively (*Figure 4*). In

Patients / Variants (%)	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20
ERBB2(p.A775_G776insYVMA)	0.76																			
EGFR(p.G719C)												0.15								
EGFR(p.G719S)				0.11			0.83									0.3		0.63		
EGFR(p.G719A)							0.1													
EGFR(p.L858R)			1.24						2.82	0.2		0.23				0.98		24.9	0.2	
EGFR(p.T790M)						1.06			1.7					6						2.6
EGFR(p.E746_A750delELREA)			0.52							3.03									3.03	
ALK(p.F1174S)													0.17							
ALK(p.L1152P)													0.11							
PIK3CA (p.H1047R)				0.22			1.22						1.07		1.04	0.34		1.06		
BRAF (p.V600E)		0.16		0.35			2.81		0.16		0.4	0.18	2.23	0.17	2.14	0.79		3.45	0.3	0.2
KRAS (p.G12A/V)													2.69		5.19					
KRAS(p.Q61R)																0.23				
KRAS(p.G12R/D)			0.39		5.16			0.18								1.26	0.23		0.1	
MAP2K1 (p.Q56P)				0.26									0.7		0.99	0.3		0.98		
MAP2K1 (p.P124S)															0.93					
NRAS(p.G13D)							0.21													
TP53 (p.Y205C)													0.1							

Figure 2 Plasma ctDNA mutations detected in 20 NSCLC patients. ctDNA, circulating tumor DNA; NSCLC, non-small cell lung cancer.

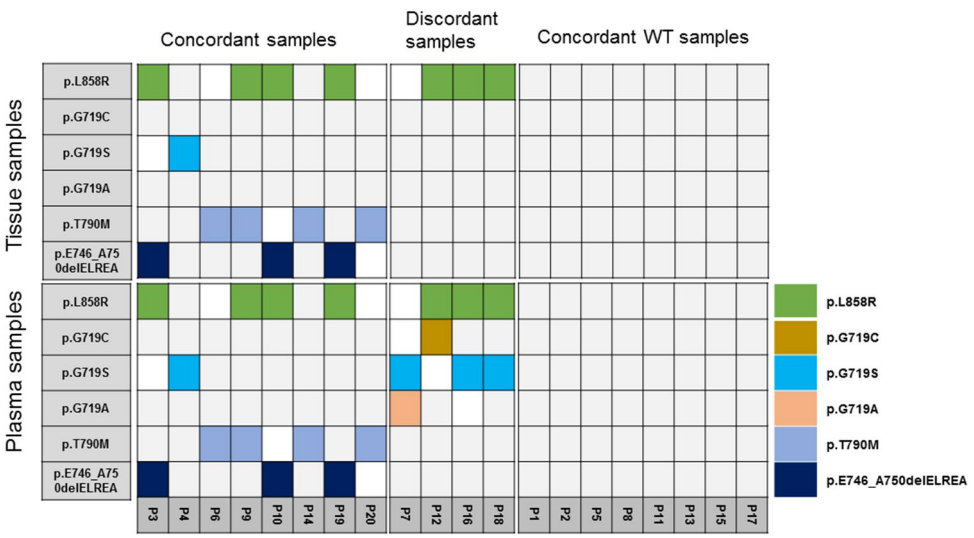

Figure 3 EGFR-related mutations in the matched plasma ctDNA and tissue DNA of 20 NSCLC patients. ctDNA, circulating tumor DNA; EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; WT, wild type.

summary, the concordant results between tag sequencing and the routine clinical approaches demonstrated that the tag sequencing method using the ctDNA has great potential in detecting gene mutations in NSCLC patients.

Discussion

In the plasma and the matched tissue samples obtained from 20 NSCLC patients, tag sequencing was applied to simultaneously detect multiple NSCLC-related gene mutations. Apart from the NSCLC samples, four different concentrations of a reference standard cfDNA set were used to evaluate the assay performance. For the NSCLC samples, the *EGFR* mutation concordance between the plasma ctDNA and tissue DNA was 80% and the sensitivity and specificity were 67% and 100%, respectively (*Figure 4*). For

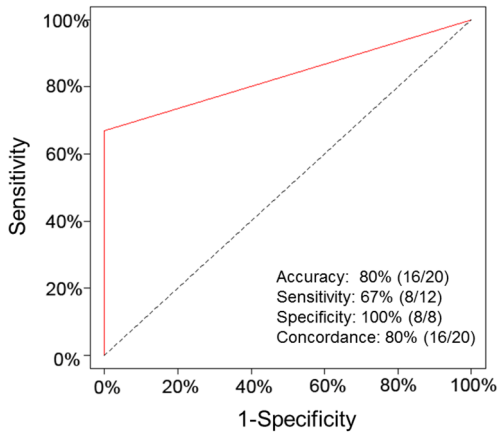

Figure 4 Comparison of the EGFR mutations in the plasma ctDNA and tissue DNA. EGFR, epidermal growth factor receptor; ctDNA, circulating tumor DNA.

the reference standard cfDNA samples, the assay detected 100% of the six known mutations, and their detection frequencies were all close to the expected allele frequencies (*Figure 1*). The assay was very stable and the SDs of all mutations were less than 0.3% in the six replicated experiments (*Table S3*). These results indicate NGS has the great potential in detecting the gene mutations of tumors, so tag sequencing could be a promising tool in NSCLC diagnosis and personalized medicine.

The genetic profiles characterized by ctDNA can monitor tumor genetic alterations to guide patient treatment. Clinical testing for *EGFR* mutations is now a routine in case of NSCLC to guide treatment. NSCLC patients with p.L858R mutations tend to be sensitive to *EGFR*-TKIs (18,19),whereas the patients with the secondary p.T790M mutation tend to develop drug resistance to the first generation of *EGFR*-TKIs (20,21). In our study, both of these mutations could be detected in the plasma ctDNA (*Figure 3*). Patients P9 with both mutations, had developed resistance to treatment with *EGFR*-TKIs, such as gefitinib and erlotinib (*Figure 4*). For the patient P3, P10, P12, P16, P18 and P19 , the plasma ctDNA could be applied to monitor whether the patients had developed the drug resistance (p.T790M) to EGFR-TKIs. Taken together, these findings indicate that the identification of ctDNA mutations has great benefits for tumor diagnosis, patient treatment and survival prediction.

Various methods have been developed to detect the mutations in the plasma ctDNA, such as PCR-based methods and NGS-based methods. The sensitivity of *EGFR* mutations detected in the ctDNA is 66.7% (34/51) by PNA-PCR (22), 72.1% (44/61) by ARMS (23) and 70.6% (12/17) by the NGS based on the hybrid capture method (15). In our study, we applied tag sequencing based on Ion Torrent to detect mutations in ctDNA. The sensitivity of *EGFR* mutations was approximately 70%, which was similar to other methods, but our NGS-based method can simultaneously detect a large number of gene mutations compared with PCR-based methods. Moreover, compared with the NGS-based hybrid capture method, our method is much less expensive and time consuming, making the technique more suitable for clinical use.

Although we found that tag sequencing accurately detected the mutations in the reference standard cfDNA set, and we observed high levels of concordance between plasma ctDNA and the matched tissue DNA, tour study had two major limitations. First, we assessed concordance merely using the *EGFR* mutations in 20 matched tissue and plasma samples. In future research, more genes, such as *BRAF*, *KRAS*, *NRAS*, *PIK3CA*, *MAP2K1* and *TP53*, should be included to comprehensively investigate the concordance between plasma ctDNA and matched tissue DNA. Also, a larger sample size will be required to further assess the performance of our concordant analysis. Third, more reference mutations were required to assess the performance of our assay. In this study, we evaluated the performance of tag sequencing using the four concentrations of the reference standard cfDNA set containing six detected mutations. Although detection was successful with four reference standard cfDNA concentrations and six replicated experiments at 0.1%, more mutations should be included to evaluate assay performance in the future.

In summary, the tag sequencing method can effectively and stably detect gene mutations in four concentrations of a reference standard cfDNA set. Moreover, our results revealed the high concordance rates between plasma ctDNA and matched tissue DNA in 20 advanced NSCLC patients, suggesting that the ctDNA in plasma has great potential in characterizing the genetic profiles of tumors, so its application may be of great value in molecular diagnostics, prognosis prediction and targeted drug selection.

Conclusions

Our findings indicated that tag sequencing can effectively and stably detect gene mutations in ctDNA, and is suitable for clinical application. Tag sequencing accurately detects the ctDNA mutations, so its application may be of great

value in molecular diagnostics, prognosis prediction and targeted drug selection.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2017.08.03). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study protocol was approved by the Ethical and Protocol Review Committee of Zhujiang Hospital. All procedures performed in studies involving human participants were in accordance with the ethical standards of Zhujiang hospital and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from all patients.

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Supplementary

Table S1 DNA concentration of each patient

Patient	ctDNA concentration (ng/μL)	Tissue DNA concentration (ng/μL)
P1	0.684	30.6
P2	0.708	70.4
P3	0.580	36.2
P4	0.718	25.0
P5	0.584	16.2
P6	0.972	34.2
P7	0.418	22.8
P8	0.454	43.4
P9	0.472	21.8
P10	0.452	28.2
P11	0.480	27.4
P12	0.594	58.4
P13	0.554	35.8
P14	0.302	37.4
P15	0.702	23.0
P16	0.258	21.8
P17	0.442	17.2
P18	0.282	30.4
P19	1.850	51.2
P20	0.438	19.5

ctDNA, circulating tumor DNA.

Table S2 EGFR mutations screened by ARMS-PCR

Cosmic ID	Mutation	Nucleotide change
6252	G719S	2155G>A
6253	G719C	2155G>T
6239	G719A	2156G>C
6223	E746-A750del [1]	2235-2249 del 15
13551	E746-T751>I	2235-2252>AAT (complex)
6225	E746-A750del [2]	2236-2250 del 15
12728	E746-T751del	2236-2253 del 18
12384	E746-S752>V	2237-2250>T (complex)
12678	E746-T751>A	2237-2251 del 15
12367	E746-S752>A	2237-2254 del 18
12422	L747-A750>P	2238-2248>GC (complex)
12419	L747-T751>Q	2238-2252>GCA (complex)
6220	E746-S752>D	2238-2255 del 18
6218	L747-E749del	2239-2247 del 9
12382	L747-A750>P	2239-2248 TTAAGAGAAG>C (complex)
12383	L747-T751>P	2239-2251>C (complex)
6254	L747-T751del	2239-2253 del 15
6255	L747-S752del	2239-2256 del 18
12387	L747-P753>Q	2239-2258>CA (complex)
6210	L747-T751>S	2240-2251 del 12
12369	L747-T751del	2240-2254 del 15
12370	L747-P753>S	2240-2257 del 18
6241	S768I	2303 G>T
12376	V769-D770insASV	2307-2308 ins (GCCAGCGTG)
12378	D770-N771insG	2310-2311 ins GGT
12377	H773-V774insH	2319-2320 ins CAC
6240	T790M	2369 C>T
6224	L858R	2573 T>G
6213	L861Q	2582 T>A

ARMS, amplification refractory mutation system; PCR, polymerase chain reaction; EGFR, epidermal growth factor receptor.

Table S3 The frequency of detected variants in the replicated 1% cfDNA

Replicates(%)	p.E545K	p.G12D	p.V769_D770insASV	p.L858R	p.T790M	p.E746_A750delELREA
1	0.91	1.51	0.70	1.46	0.71	1.01
2	1.33	1.47	0.82	0.84	0.90	1.35
3	1.24	1.04	0.85	0.80	0.62	0.95
4	1.00	1.31	0.87	1.05	0.92	1.15
5	1.21	1.25	0.75	0.63	0.82	1.22
6	1.04	1.31	0.85	0.81	0.90	0.98
Mean	1.21	1.32	0.81	0.93	0.81	1.10
Standard deviation	0.16	0.17	0.07	0.3	0.12	0.16
Expected frequency	1.30	1.30	1.00	1.00	1.00	1.00

cfDNA, cell free DNA.

Table S4 The performance of mutation detected in plasma ctDNA

Mutations	Sensitivity (%)	Specificity (%)	Concordance (%)
p.L858R	100 (7/7)	100 (13/13)	100 (20/20)
p.G719X	17 (1/6)	100 (54/54)	92.7 (55/60)
p.T790M	100 (4/4)	100 (16/16)	100 (20/20)
p.E746_A750delELEREA	100 (3/3)	100 (17/17)	100 (20/20)
In total	66.7 (8/12)	100 (8/8)	80 (16/20)

ctDNA, circulating tumor DNA.