



The clinical value of circulating tumor DNA detection in advanced non-small cell lung cancer

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Background: Circulating tumor DNA (ctDNA) is a kind of cell-free DNA which comes from tumor cells and effectively reflects the molecular characteristics of tumors, which providing us a novel method to explore its clinical therapeutic value in advanced lung cancer.

Methods: A total of 36 patients with advanced non-small cell lung cancer (NSCLC) were enrolled in this study, including 28 cases of adenocarcinoma and 8 cases of squamous cell carcinoma. Next-generation sequencing based ctDNA detection, tissue DNA (tDNA) detection, corresponding survival analysis, and retrospective statistics were performed to explore the feasibility of clinical practice directed by molecular characteristics in NSCLC.

Results: Epidermal growth factor receptor mutation (*EGFR* mutation) took over the highest mutation frequency (36.11%) in 36 samples, and the subsequent genes were *PIK3CA*, *BRAF*, *KRAS*, *NRAS*, *MAP2K1*, and *GNAQ*; 11 patients were detected with multiple gene mutations, including 8 cases with double gene mutations, 1 case with three gene mutations, and 2 cases with four gene mutations, and the subsequent 12-month survival observation revealed that patients with less mutations also had a longer OS (10.37±0.74 vs. 7.08±1.43 months, P=0.034). Twenty-one patients with *EGFR* mutation and subsequently treated with *EGFR*-tyrosine kinase inhibitor (TKI) combined chemotherapy, had significantly longer PFS than those with *EGFR* wild type and treated with chemotherapy in next 5-year monitoring test (18.00±4.41 vs. 7.33±1.58 months, P=0.024).

Conclusions: Gene mutation in advanced lung cancer is complex, and ctDNA detection has important guiding significance in clinical treatment of advanced NSCLC.

Keywords: Non-small cell lung cancer (NSCLC); circulating tumor DNA (ctDNA); next-generation sequencing; epidermal growth factor receptor mutation (*EGFR* mutation)

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Introduction

With the highest mortality among all cancer-related deaths, lung cancer is always a kind of highly malignant disease that severely threatens human life. Approximately 1.8 million people, 13% of all new cancer cases were diagnosed to

be lung cancer in 2012 worldwide (1). The morbidity of lung cancer in China is also belonging to a high incidence rate. According to the latest investigations, there were 0.73 million new patients and 0.61 million deaths due to lung cancer in China every year, and indicating that

lung cancer has become the leading killer of malignant tumors in China (2). Lung cancer includes non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) according to its histological and pathological characteristics, and NSCLC accounts for 80% to 85% of all lung cancer. Metastasis in NSCLC patients always represent a poor prognosis, with a median survival of 3 to 6 months (3). To our disappointment, 10–25% of the newly diagnosed advanced NSCLC patients are commonly accompanied with metastasis, and even in those with orthotopic tumor, 20–40% would develop into brain metastasis and 30–40% would develop into bone metastasis in the following treatment (4–7).

The conventional therapy for NSCLC includes operation, chemotherapy, radiotherapy, and targeted therapy, combined treatment and multi-line drugs are chosen to prolong the survival of advanced cohorts. However, there is significant difference appeared in disease progression and drug resistance among NSCLC individuals, which suggesting that it is necessary to recognize the various characteristics of NSCLC from different perspectives, such as molecular marker or DNA marker (8). The circulating tumor DNA (ctDNA), existing in peripheral blood, is a kind of DNA fragment comes from tumor cells and carries molecular mutational information (including single nucleotide variant, deletions, insertions, rearrangements, copy number variants, and methylation, etc.) of different cells, such as necrotic, apoptotic tumor cells, circulating tumor cells, and exosomes secreted by tumor cells (9). As a dynamic biomarker of tumor cells, a series of remarkable discoveries indicated ctDNA to be valuable in disease progression and drug resistance in advanced NSCLC (10–12). For example, ctDNA detection revealed that epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) drugs resistance chiefly derived from the T790M mutation (threonine to methionine substitution at codon 790) in EGFR, and the aberrations of MET was associated with poor prognosis (13,14).

In this study, high-throughput sequencing technology and bioinformatic analysis were used to explore mutational spectrum of the ctDNA which was extracted from peripheral blood of patients with advanced NSCLC. We characterized the molecular features of post-treatment patients and analyzed the correlation of genetic mutation and prognosis, and results may serve as the reference of subsequent precision medicine, accurate illness monitoring and prognosis.

Methods

Patient cohorts and ethics statement

This subject recruited the cancer patients who underwent ctDNA detection in Zhejiang Cancer Hospital from January 2017 to October 2017, and out of which 36 patients presenting with NSCLC in stage IIIB–IVB (International Union Against Cancer criteria) were eventually enrolled in study.

All procedures in this study had been approved by the Ethics Committee of Zhejiang Cancer Hospital (IRB-2018-201), and the methods were carried out in accordance with the approved guidelines. All patients involved in this research provided written informed consent for the use of blood samples, and the application of clinical medication was under the approval of the Ethics Committee.

Samples collection and DNA extraction

Streck blood collection tubes were used to collect 10 mL peripheral blood of every patient enrolled. The tubes were gently turned upside down for blending at least 10 times, and then were stored at 6–25 °C until use. For surgical tissue samples, the total mass of each sample was required no less than 60 mg, the proportion of tumor cells was required no less than 70%, and the proportion of necrotic cells was required no more than 10%. No less than 3 needles were required for percutaneous puncture samples. All tissue samples were stored in DNA preservation tubes (1Gene, Hangzhou, China) and were handled in 2 days.

DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) was used to extract cell free DNA and genomic DNA from blood samples and tissue samples respectively. Agarose gel electrophoresis was performed to test the degradation and protein/RNA pollution of extracted DNA. Concentration of extracted DNA was determined by Qubit dsDNA HS assay kit on Qubit Fluorometer according to the manufacturing protocol (Life Technologies, Carlsbad, CA, USA). DNA quality (OD 260/280) was measured by Nanodrop (Thermo Fisher Scientific, USA). The extracted DNA sample which had an OD ratio between 1.8 and 2.0 and a mass more than 10 ng was considered qualified and would be used for following library construction.

Library preparation and target-hybrid capturing

Library preparation was performed as the manufacturer's instructions of KAPA Hyper prep kit (Illumina Co.,

Table 1 Clinical characteristics of enrolled patients (n=36)

Characteristics	Parameter value	Percentage value, %
Age, years	Median: 62	Range: 46–74
Gender		
Male	18	50.0
Female	18	50.0
Smoking history		
Yes	17	47.2
No	19	52.8
Pathological diagnosis		
Adenocarcinoma	28	77.8
Squamous cell carcinoma	8	22.2
Pathological stage		
IIIB	4	11.1
IVB	32	88.9
Total	36	100.0

Ltd, San Diego, USA). In short, the fragmental DNA was respectively subjected to terminal repair, A-tailing adenylation, and ligation to indexed adapters. The targeted sequencing is a custom hybridization capture based assay which was designed by 1Gene, Inc., (Hangzhou, China). After library construction and hybridization capture, the targeted DNA fragments were amplified by polymerase chain reaction.

Sequencing and bioinformatic processing

PE150 sequencing was performed on Illumina CN500 platform. The quality control of raw data was conducted, and the clean reads were obtained after removing adaptor sequences and low mapping quality reads. For variant analysis, sequences were aligned to the reference human genome (hg19) with BWA software. The linkage of sequencing fragments was optimized by GATK, and the annotation of SNP/Indel was acquired with VarScan2. The sequencing coverage of targeted positions was required $\geq 90\%$, and the average sequencing depth of ctDNA samples and tDNA samples was respectively required $\geq 1,000\times$ and $\geq 500\times$. A variant with a variant frequency $\geq 0.3\%$ for ctDNA samples or $\geq 1.0\%$ for tDNA samples was considered credible. Final results were filtered through 1,000 genomes

database and dbSNP database.

Statistical analysis

All data were statistically analyzed by statistical software SPSS 20.0. Measurement data was exhibited as mean \pm standard deviation (SD). The relationship between mutational genes and clinicopathology was measured by Fisher's exact or Chi-square tests as appropriate. Log-rank test was used for survival analysis. Two-sided $P < 0.05$ was considered as statistical significance.

Results

Patient characteristics

The clinicopathological characteristics of enrolled patients are presented in *Table 1*. This study totally recruited 36 NSCLC patients with median age of 62 years old (range, 46 to 74 years old), including 18 males and 18 females. According to the histological classification of lung cancer published by World Health Organization, 28 patients were diagnosed as adenocarcinoma and 8 were squamous cell carcinoma; 17 patients had smoking histories. There were 32 patients in stage IVB and 4 patients in stage IIIB.

In the total 36 patients, 9 patients were untreated and received ctDNA detection before their primary treatment, and other 27 patients were re-treated and received ctDNA detection when their disease began to progress in therapeutic process. Out of the 27 re-treated patients, 21 patients had ever received tissue DNA (tDNA) detection at baseline. The schematic of enrolled patients is showed in *Figure 1*.

Actionable aberrations

Following with the deep hybrid-captured sequencing, we filtered the patients' variants with a gene database which was established by 1Gene Bio-tech Co., Ltd. and contained 66 drug-associated genes in lung cancer. Corresponding results are exhibited in *Table 2*, totally 43 actionable aberrations were discovered in 27 patients (75%, 27/36, 22 were re-treated and 5 were untreated), including 21 cases of adenocarcinoma and 6 cases of squamous cell carcinoma. Gene EGFR had the highest mutational frequency with 36.1% (13/36), and the subsequent mutational genes were PTEN (13.9%, 5/36), MAP2K1 (11.1%, 4/36), KRAS (11.1%, 4/36), PIK3CA (8.3%, 3/36), GNAQ (5.6%, 2/36) and BRAF (5.6%, 2/36). When considering pathological

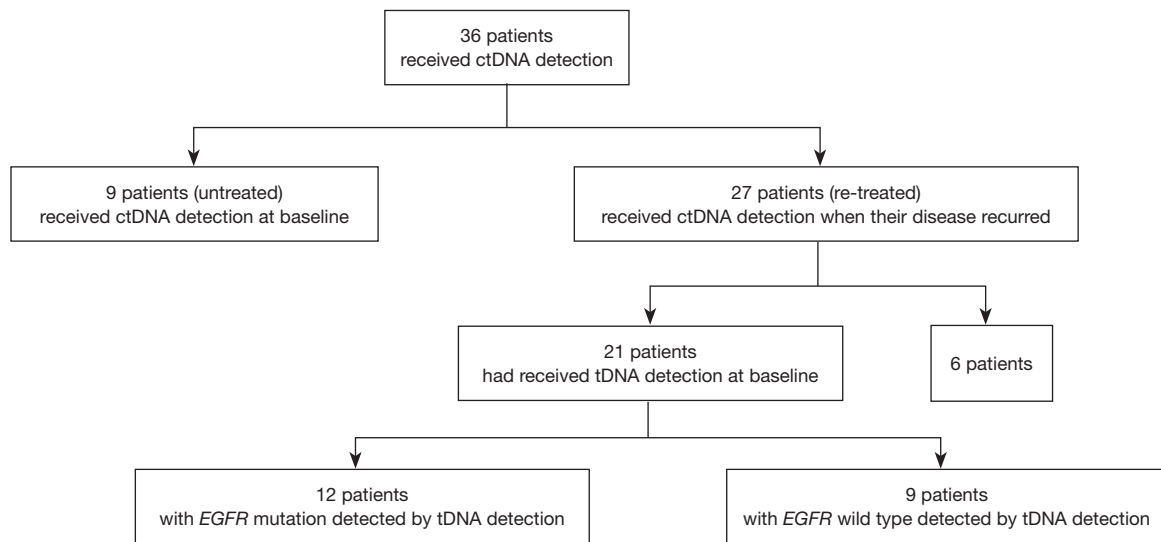


Figure 1 Schematic of enrolled patients. ctDNA, circulating tumor DNA; tDNA, tissue DNA.

Table 2 Actionable mutations identified by ctDNA detection (n=36)

Patient ID	Gene	c.anno	p.anno	Pathological type	Treatment status	Subsequent treatment
10267	<i>EGFR</i>	c.2573T>G	p.L858R	AC	RE	Palliative care
10395	<i>EGFR</i>	c.2236_2250del15	p.E746_A750delELREA	AC	RE	TKI
10422	<i>PTEN</i>	c.697C>T	p.R233*	AC	RE	Chemotherapy
10430	<i>KRAS</i>	c.38G>A	p.G13D	AC	RE	Palliative care
10506	<i>EGFR</i>	c.2369C>T	p.T790M	AC	RE	TKI
10507	<i>MAP2K1</i>	c.167A>C	p.Q56P	AC	RE	TKI
10512	<i>MAP2K1</i>	c.157T>C	p.F53L	AC	RE	TKI
10551	<i>GNAQ</i>	c.548G>A	p.R183Q	SC	RE	Palliative care
10783	<i>PIK3CA</i>	c.3140A>T	p.H1047L	SC	RE	TKI
10789	<i>KRAS</i>	c.34G>A	p.G12S	AC	RE	Palliative care
10823	<i>EGFR</i>	c.2236_2250del15	p.E746_A750delELREA	AC	RE	TKI
10864	<i>PIK3CA</i>	c.1624G>A	p.E542K	AC	RE	Palliative care
10877	<i>EGFR</i>	c.2573T>G	p.L858R	AC	RE	TKI
11085	<i>EGFR</i>	c.2240_2254del15	p.L747_T751delLREAT	AC	RE	TKI
10836	<i>EGFR</i>	c.2236_2250del15	p.E746_A750delELREA	AC	UN	†
10040	<i>PTEN</i>	c.389G>A	p.R130Q	SC	UN	†
10184	<i>KRAS</i>	c.182A>T	p.Q61L	AC	RE	Chemotherapy
	<i>PTEN</i>	c.388C>T	p.R130*			
10257	<i>EGFR</i>	c.2573T>G	p.L858R	AC	RE	TKI
	<i>EGFR</i>	c.2369C>T	p.T790M			

Table 2 (continued)

Table 2 (continued)

Patient ID	Gene	c.anno	p.anno	Pathological type	Treatment status	Subsequent treatment
10312	<i>BRAF</i>	c.1799T>A	p.V600E	AC	RE	Palliative care
	<i>PTEN</i>	c.388C>T	p.R130*			
10792	<i>EGFR</i>	c.2582T>A	p.L861Q	SC	RE	TKI
	<i>EGFR</i>	c.2369C>T	p.T790M			
10843	<i>EGFR</i>	c.2369C>T	p.T790M	AC	RE	TKI
	<i>EGFR</i>	c.2240_2257del18	p.L747_P753delinsS			
10415	<i>EGFR</i>	c.2573T>G	p.L858R	AC	RE	TKI
	<i>EGFR</i>	c.2369C>T	p.T790M			
11088	<i>KRAS</i>	c.35G>T	p.G12V	AC	RE	TKI
	<i>MAP2K1</i>	c.199G>A	p.D67N			
11095	<i>GNAQ</i>	c.548G>A	p.R183Q	SC	RE	Palliative care
	<i>PTEN</i>	c.697C>T	p.R233*			
11353	<i>EGFR</i>	c.2155G>A	p.G719S	AC	UN	†
	<i>EGFR</i>	c.2582T>A	p.L861Q			
	<i>KRAS</i>	c.37G>A	p.G13S			
10064	<i>BRCA1</i>	c.3548A>G	p.K1183R	SC	UN	†
	<i>BRCA1</i>	c.2612C>T	p.P871L			
	<i>KIT</i>	c.1676T>G	p.V559G			
	<i>MAP2K1</i>	c.332T>G	p. I111S			
10558	<i>BRAF</i>	c.1397G>T	p.G466V	AC	UN	†
	<i>EGFR</i>	c.2235_2249del15	p.E746_A750delELREA			
	<i>HRAS</i>	c.182A>G	p.Q61R			
	<i>PIK3CA</i>	c.1624G>A	p.E542K			
10273	-	-	-	AC	UN	†
10423	-	-	-	AC	UN	†
11103	-	-	-	SC	UN	†
10248	-	-	-	AC	UN	†
10271	-	-	-	AC	RE	TKI
11106	-	-	-	SC	RE	Palliative care
11097	-	-	-	AC	RE	Chemotherapy
10921	-	-	-	AC	RE	TKI
10503	-	-	-	AC	RE	TKI

AC, adenocarcinoma; SC, squamous cell carcinoma; RE, re-treated; UN, untreated; c.anno, cDNA annotation; p.anno, protein annotation; *, nonsense mutation; -, negative; †, the treatment of newly diagnosed patients were not included.

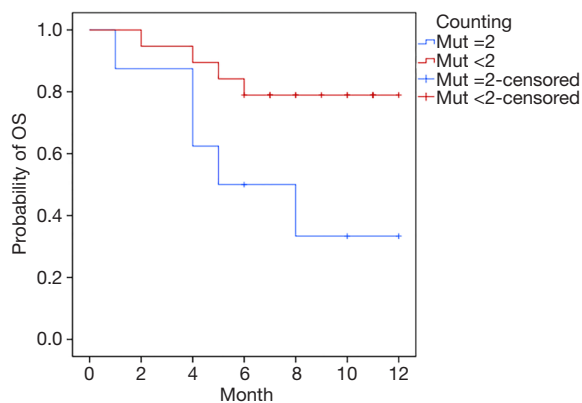


Figure 2 OS of the recurrent patients with “mutation count =2” (blue line) and “mutation count <2” (red line) identified by ctDNA detection (7.08 ± 1.43 vs. 10.37 ± 0.74 months, $P=0.034$, $n=27$). OS, overall survival; mut, mutation.

type, *EGFR* was also the most frequent mutational gene in the 28 patients with adenocarcinoma (42.86%, 12/28), followed with *KRAS* (17.86%, 5/28), *MAP2K1* (10.71%, 3/28), *PTEN* (10.71%, 3/28), *PIK3CA* (7.14%, 2/28), *BRAF* (7.14%, 2/28), *HRAS* (3.57%, 1/28). In the 8 patients with squamous cell carcinoma, the most frequent mutations were *PTEN* and *GNAQ*, with the same rate 25% (2/8), and *EGFR*, *PIK3CA*, *KIT* also had the same variant frequency of 12.5% (1/8). None of patients was detected with the mutated fusion in gene *ALK* or *ROS1* in this study.

The phenomenon of multiple mutations was discovered in 11 patients, including 8 patients with double gene mutations, 1 patient with three gene mutations, and 2 patients with four gene mutations. Among these patients, we observed that aberrations usually appeared with an accompaniment of *EGFR* mutation (55%, 6/11), and we speculated that *EGFR* mutation may be one of the primary founding clusters of lung cancer. Twenty-two out of 27 re-treated patients (81.5%, 22/27) were identified with pathogenic mutations, and all the mutational genes are located in the protein kinase signaling pathways. Furthermore, 5 patients were characterised with the *EGFR* p.T790M aberration which is known as the main cause of tyrosine kinase inhibitor (TKI) drug resistance in NSCLC. In summary, ctDNA detection technology provides us with a novel helpful method to study the pathogenic mechanisms, drug resistance, and disease recurrence in NSCLC.

It was on the time when disease began to progress that the 27 re-treated patients received ctDNA detection. Results pointed out that 8 patients had double-gene

mutations (mutation count =2) and 19 patients had non-gene mutation or single-gene mutation (mutation count <2) (Table 2). Subsequent therapeutic strategy was partially based on the genomic information. The survival follow-up was continuing to April 30, 2018, and it showed that a total of 9 patients were dead, including 5 patients in group “mutation count =2” and 4 patients in group “mutation count <2”. The 12-month survival rate of group “mutation count =2” and group “mutation count <2” was 50.00% (4/8) and 84.21% (16/19) respectively, and there was a statistically significant difference between the two groups (7.08 ± 1.43 vs. 10.37 ± 0.74 months, $P=0.034$; Figure 2).

Correlation between *EGFR* status and clinicopathologic characteristics

The correlation between mutational status of *EGFR* and clinicopathologic features was analyzed. Results showed that *EGFR* mutations usually occurred in younger patients in this study, the positive mutational rate of patients aged under 60 and above 60 was 57.1% and 22.7% respectively ($P<0.05$). There were no statistical differences between *EGFR* status and other clinical features, such as gender, pathological diagnosis, smoking history, pathological stage, and primary treatment (Table 3).

In the process of analysis, we also found that all the squamous cell carcinoma patients (100%, 8/8) and 47.4% (9/19) adenocarcinoma patients had smoking history. There was a significant relation between pathological diagnosis and smoking history ($P<0.001$), indicating that people who have smoking habit are more likely to suffer squamous cell carcinoma than adenocarcinoma.

EGFR targeted therapy

We also compared the differences of mutational characteristics between ctDNA detection and tDNA detection among the 21 patients who had received a tDNA detection at baseline. It was found that the mutational characteristics had changed in the majority of patients (85.7%, 18/21) from baseline to disease progression (Table 4). In the 9 patients who had been classified as *EGFR*-wt (wild type) by tDNA detection, 8 patients (88.9%, 8/9) were detected out of genomic mutations by subsequent ctDNA detection, including 3 with *EGFR* mutations (33.3%, 3/9), 3 respectively with *KRAS*, *PIK3CA*, *MAP2K1* mutation (11.1%, 1/9), 1 with *BRAF*, *PTEN* double-gene mutations (11.1%, 1/9), 1 with *KRAS*, *PTEN* double-gene mutations

Table 3 Correlation between *EGFR* status and clinical characteristics (n=36)

Variables	<i>EGFR</i> -mut	<i>EGFR</i> -wt	P
Age, years			0.036
<60	8	6	
≥60	5	17	
Gender			0.298
Female	8	10	
Male	5	13	
Pathological diagnosis			0.093
AC	12	16	
SC	1	7	
Smoking history			0.137
Ever	4	13	
Never	9	10	
Pathological stage			1.000
IIIB	1	3	
IVB	12	20	
Primary treatment			1.000
Yes	3	6	
No	10	17	

AC, adenocarcinoma; SC, squamous cell carcinoma; mut, mutation; wt, wild type.

(11.1%, 1/9). In the 12 patients who were primary *EGFR*-mut (mutation) confirmed by tDNA detection, 3 were detected with non-mutations (25%, 3/12), 4 were found accompanying with a new mutational loci p.T790M in *EGFR* (33.3%, 4/12), 3 had different mutational genes (25%, 3/12), 2 were the same as primary (16.7%, 2/12), according to the ctDNA detection (Table 4).

Based on the tDNA detection, we classified the 21 patients into two groups according to the status of *EGFR*. The subsequent therapeutic strategies were adopted according to the classification, 12 patients in group *EGFR*-mut were subsequently treated with *EGFR*-TKI or *EGFR*-TKI combined chemotherapy (some patients gave up TKI halfway because of poor tolerance or costly expense and adopted chemotherapy alternatively), and other 9 patients in group *EGFR*-wt were treated with platinum chemotherapeutic drugs (Table 4). The comparative analysis of PFS (progression free survival) between the two groups in

the next 5 years revealed that patients with *EGFR*-mut had a significantly longer stable stage than those with *EGFR*-wt (18.00±4.41 vs. 7.33±1.58 months, P=0.024) (Figure 3).

Discussion

NSCLC is the most common type and accounting for 80% of lung cancer, and the two main pathological classifications of NSCLC are adenocarcinoma and squamous cell carcinoma (15). Tissue biopsy is the gold standard for the diagnosis of lung cancer in the current mainstream views. However, with the development of liquid biopsy technology, ctDNA detection, which was used as a complementary test for tDNA detection, has become more and more clinically significant for patients whose tissue samples were unobtainable (16). CtDNA is a kind of cell-free DNA which comes from the genome of tumor cells and circulating in peripheral blood. A couple of studies have proven that ctDNA is of great concordance with tumor tissue DNA in sensitivity and specificity of tumor associated DNA mutations (17-19).

In this study, deep targeted sequencing was performed to reveal the gene mutations of 36 advanced NSCLC patients by their ctDNA samples. Results indicated that 27 patients had at least one mutational gene, and the most common mutational gene was *EGFR*, with a frequency of 36.11%, including 42.86% in patients with adenocarcinoma and 12.5% with squamous cell carcinoma. Our finding is in consistent with previous studies which reported that the mutational frequency of *EGFR* in NSCLC detected in ctDNA ranges from 20.4% to 43.0% (20,21).

The total mutational frequency of aberrations in *EGFR* was the highest among all the therapeutic genetic targets in Oriental population. Previous studies found that the mutational frequency of *EGFR* in lung adenocarcinoma range from 47.9% to 55.7%, while it is much lower in lung squamous cell carcinoma (22). Similarly, our result also showed that the mutational frequency of *EGFR* in adenocarcinoma and squamous cell carcinoma was 42.86% and 12.5% respectively. In this study, *EGFR* mutations usually occurred in younger patients with age ≤60 (P<0.05), and there was no statistical significance in *EGFR* status and other clinical features, which was different from other studies probably because of the limited sample size (23,24). At the same time, our analysis proved that smoking had significant statistical correlation with pathological type (P<0.001) in advanced NSCLC, indicating that smokers are more likely to develop into squamous cell carcinoma but

Table 4 Mutation status of patients received ctDNA detection (n=21)

Patient ID	Mutational status		Using targeted drugs	Using chemotherapeutic drugs	Time to progression (month)
	At baseline	At disease progression			
10257	<i>EGFR</i> L858R	<i>EGFR</i> L858R; <i>EGFR</i> T790M	Y	Y	8
10843	<i>EGFR</i> Ex19 Del	<i>EGFR</i> Ex19 Del; <i>EGFR</i> T790M	Y	Y	3
10415	<i>EGFR</i> L858R	<i>EGFR</i> L858R; <i>EGFR</i> T790M	Y	Y	31
10506	<i>EGFR</i> Ex19 Del	<i>EGFR</i> T790M	Y	N	14
11088	<i>EGFR</i> Ex19 Del	<i>KRAS</i> G12V; <i>MAP2K1</i> D67N	Y	Y	53
10512	<i>EGFR</i> Ex19 Del	<i>MAP2K1</i> F53L	Y	N	7
10422	<i>EGFR</i> L858R	<i>PTEN</i> R233*	Y	N	38
10823	<i>EGFR</i> Ex19 Del	<i>EGFR</i> Ex19 Del	Y	N	7
10877	<i>EGFR</i> Ex19 Del; <i>EGFR</i> L858R	<i>EGFR</i> L858R	Y	Y	14
10503	<i>EGFR</i> Ex19 Del	-	Y	N	16
10271	<i>EGFR</i> Ex19 Del	-	Y	Y	5
10921	<i>EGFR</i> G719X	-	Y	Y	20
11106	-	-	N	Y	8
10864	-	<i>PIK3CA</i> E542K	N	Y	16
10267	-	<i>EGFR</i> L858R	N	Y	5
10430	-	<i>KRAS</i> G13D	N	Y	2
10312	-	<i>BRAF</i> V600E; <i>PTEN</i> R130*	N	Y	12
10184	-	<i>KRAS</i> Q61L; <i>PTEN</i> R130*	N	Y	11
10395	-	<i>EGFR</i> Ex19 DEL	N	Y	3
11085	-	<i>EGFR</i> Ex19 DEL	N	Y	4
10507	-	<i>MAP2K1</i> Q56P	N	Y	5

*, nonsense mutation; -, negative; Ex, exon; Del, deletion; N, no; Y, yes.

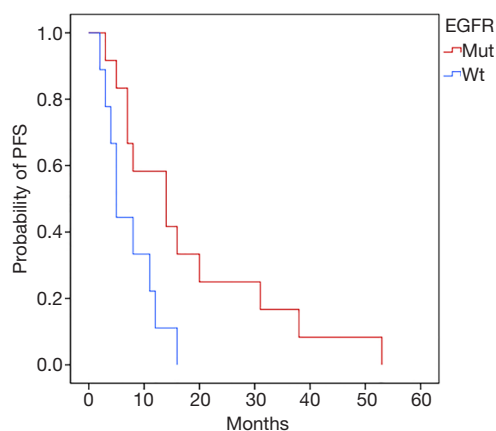


Figure 3 PFS of *EGFR*-mut patients treated with *EGFR*-TKI (red line) and *EGFR*-wt patients treated with chemotherapy (blue line) (18.00 ± 4.41 vs. 7.33 ± 1.58 months, $P=0.024$, $n=21$). PFS, progression-free survival; mut, mutation; wt, wild type.

not adenocarcinoma.

Multiple mutations were rarely reported in previous clinical studies of lung cancer, and the majority of reported mutational frequency ranged 3.13% to 8.33% (25,26). There were 11 out of 36 patients in this study had been detected with multiple mutations, with a frequency of 30.56%, which was higher than previous studies. It was speculated that the enrolled 36 patients were all advanced cases and most of them underwent disease progression and drug resistance after primary treatment, which might lead to the appearance of new mutations. On the other hand, limited sample size and the biased composition of pathological type might also lead to the different outcomes.

The results of ctDNA detection which performed to the 27 re-treated patients showed that 8 patients were classified as double-gene mutations and 19 patients were single-

gene mutation or negative mutation. Subsequently clinical medication was also determined partially according to the genomic status, and the overall survival of patients with double-gene mutations was significant shorter than patients with single-gene mutation or non-gene mutation ($P=0.034$), suggesting that ctDNA may serve as one of the indicators of prognosis in the future.

The survival analysis of the 21 patients who were detected with *EGFR* mutations by tissue biopsy revealed that *EGFR*-mut patients treated with *EGFR*-TKI or *EGFR*-TKI combined chemotherapy had a significantly longer progression free survival than *EGFR*-wt patients treated with chemotherapeutics only ($P=0.024$). Therefore, the detection of *EGFR* mutation in NSCLC patients showed important clinical significance during treatment sessions (27-29). Subsequently, a ctDNA detection was conducted to the above 21 patients when they showed a disease progression or drug resistance. Results revealed that the mutational spectrum had changed in the majority of them (85.7%, 18/21), and it's speculated that the changes of mutational spectrum might lead to those bad outcomes, though more validation tests need to be performed. All these results suggest us that it is useful to re-perform gene detection for the following disease monitoring and drug selection for patients who were undergoing disease progression or drug resistance.

Conclusions

In summary, this study revealed the molecular mutational characteristics of advanced NSCLC patients with post-treatment disease progression by next-generation sequencing based ctDNA detection, and proved the capability of molecular diagnosis by liquid biopsy for advanced lung cancer patients. When comparing the results between baseline tDNA detection and post-treatment ctDNA detection, we found that the genomic mutational spectrum had changed in the majority of the patients who underwent disease progression or drug resistance. Patients with multiple mutations obtained a shorter overall survival than those with less mutations, which indicates that multiple genes mutation may indicate a poor prognosis. Therefore, it is necessary to take a gene detection for therapeutic options and disease progression monitoring for NSCLC patients.

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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.2019.01.20>). 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 was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Zhejiang Cancer Hospital (IRB-2018-201), and written informed consent was obtained from all patients.

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