Am J Transl Res 2022;14(8):5605-5613
www.ajtr.org /ISSN:1943-8141/AJTR0143293

Original Article

Comparison of *EGFR* mutations detected by LNA-ARMS PCR in plasma ctDNA samples and matched tissue sample in non-small cell lung cancer patients

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Received April 3, 2022; Accepted July 6, 2022; Epub August 15, 2022; Published August 30, 2022

Abstract: Background: Screening for epidermal growth factor receptor (*EGFR*) mutations is the key to select suitable patients with non-small cell lung cancer (NSCLC) for EGFR-TKI therapy in clinical practice. Nevertheless, tumor tissue that needed for mutation analysis is frequently unavailable, especially for patients with recurrence after operation. Therefore, detection of *EGFR* from circulating tumor DNA (ctDNA) in patients with NSCLC is a sensitive and convenient method to direct patient sequential treatment strategy. Methods: One hundred and seventy-nine NSCLC patients with both tumor tissue samples and paired plasma samples were recruited. *EGFR* mutations were detected in 68 tumor tissue samples and 179 plasma samples using Anlongen Locked Nucleic Acid-Amplification Refractory Mutation System (LNA-ARMS) *EGFR* Mutation Detection Kit. The remaining 111 tumor tissue samples were detected with the use of multiplex PCR-Based NGS sequence. We calculated the sensitivity, specificity, positive prediction value (PPV) and negative prediction value (NPV) of LAN-ARMS PCR. The objective response rate (ORR) of patients received TKIs therapy was calculated. Results: Of the 179 patients, *EGFR* mutations were detected in 77 of the 179 tumor tissue samples, with a positive rate of 43.01% (77/179). In addition, *EGFR* mutations were detected in 42 of the 179 plasma samples. The sensitivity and specificity of LAN-ARMS in detecting EGFR mutations were 57.18% and 98.04% respectively compared to tissue results. The PPV was 95.24%, and NPV was 72.99%. Of the 179 pair of samples, EGFR mutations were inconsistent in 39 pairs of tissue and plasma. The overall agreement of *EGFR* mutation detection was 78.21% (140/179). The ORR was higher in patients with both tissue and plasma *EGFR* mutations compared with that in patients with only tissue *EGFR* mutations (73.33% vs. 68.29%), but the difference was not significant. It was suggested that tissue detection combined with plasma detection could improve the mutation rate. Conclusion: In plasma samples, Anlongen LAN-ARMS *EGFR* Mutation Detection Kit had a high sensitivity and specificity for the detection of *EGFR* mutations. Anlongen LAN-ARMS *EGFR* Mutation Detection Kit had the advantages of easy-to-operate and high sensitivity in clinical application.

Keywords: Locked nucleic acid-amplification refractory mutation system (LNA-ARMS), epidermal growth factor receptor (*EGFR*), lung cancer

Introduction

Lung cancer is the leading cause of cancer death worldwide, and non-small cell lung cancer (NSCLC) is the most common type comprising about 85% of all lung tumors [1]. Since symptoms of early lung cancer were inconspicuous, the majority of patients are in an advanced stage at diagnosis [2]. For patients with advanced NSCLC, chemotherapy has long been the standard therapy. But the response rate of the traditional chemotherapy was low, and the toxic and side effects were high.

Molecular target therapy of the lung cancer has been proved to have higher efficacy and less side effects than traditional chemotherapy for patients with advanced NSCLC. Epidermal growth factor receptor (*EGFR)* tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib, have been approved for patients with EGFR mutation-positive advanced NSCLC [3-6].

Therefore, *EGFR* mutation detection for future screening EGFR-TKI sensitive patients is essential [7, 8].

For *EGFR* mutations, tumor tissue measurement is the gold standard but has some limitations. First of all, for recurrent patients, the tumor tissue was not easy to get [9]. Second, there may be sampling bias in the mutation measurement from tissues due to the heterogeneity of the tumors [10]. Finally, secondary resistance of tumors triggers the practical clinical need for dynamic monitoring of *EGFR* mutations [11, 12]. However, to acquire a series of tumor tissues is difficult for the patients. Therefore, noninvasive detection methods for tumor mutations are needed.

The detection of circulating tumor DNA (ctDNA) in plasma has been applied in clinic [13-15]. There have been a variety of methods detecting *EGFR* mutations in plasma ctDNA of lung cancer patients in the past decade, such as Amplification Refractory Mutation System (ARMS), denaturing high-performance liquid chromatography, droplet digital PCR (ddPCR) and next-generation sequencing (NGS) [16-20]. Among these, ARMS is regarded as an economical, simple and rapid method suitable for clinical practice. However, when detecting *EGFR* mutations by ARMS, the sensitivity was only 48.2%-67.4%, while the specificity was as high as 93.5%-99% [19, 21, 22]. It was suggested that plasma ARMS based *EGFR* mutation detection was limited by low sensitivity. Therefore, it is necessary to develop a high sensitivity ARMS detection Kit.

In this study, a clinical study was conducted to assess the performance of this new method in detecting clinical plasma samples and predicting the therapeutic effect of EGFR-TKIs.

Materials and methods

Patient recruitment

In the present study, 229 patients with recurrent or postoperative NSCLC admitted to the Affiliated Hospital of Inner Mongolia Medical University from June 2018 to June 2020 were retrospectively enrolled. The inclusion criteria including: 1) patients with newly diagnosed or postoperative recurrent NSCLC [23]; 2) patients with adequate hematological function, coagulation, liver function and renal function. The exclusive criteria including: 1) patients who previously received targeted EGFR treatment; 2) patients with CNS metastases; 3) patients without paired tissue and plasma samples.

Patients with newly diagnosed or postoperative recurrent NSCLC without follow-up treatment were included in this study. Patients who did not have paired tissue or plasma samples or whose tissue and paired plasma samples were collected more than 14 days apart were excluded. In total, 50 patients were excluded, and 179 patients were included for later EGFR mutation analysis. The tumor tissue included 7 cases of transbronchial lung biopsy, 30 cases of malignant pleural effusion, 6 cases of fresh surgical tissue and 136 cases of paraffin section.

Tumor tissue and paired plasma samples of the 179 patients were collected. Of the 179 tissue samples, *EGFR* mutations of 111 tissue samples were detected using multiplex PCR-Based NGS sequence, and 68 tissue samples were detected by Anlongen LAN-ARMS *EGFR* Mutation Detection Kit (Anlongen Science and Technology Co. Ltd. Hefei, China). *EGFR* mutations of all 179 plasma samples were detected with the use of Anlongen LAN-ARMS *EGFR* Mutation Detection Kit.

Of the 179 patients, EGFR targeted drug was used for 41 patients with EGFR mutations. Therefore, objective response rate (ORR) of 41 patients as the response of the targeted drug was followed up after treatment. EGFR treatment efficacy was assessed based on RECIST v1.1 criteria. Complete response (CR) was defined as all the target lesions were disappearance, and no new lesion appeared with tumor markers remained normal for at least 4 weeks. Partial response (PR) was defined as the sum of the maximum diameter of target lesions was reduced by 30% for at least 4 weeks. The ORR was the rate of the patients with CR and PR after EGFR-TKIs therapy.

This study was approved by the Human Research Ethical Committee of the Affiliated Hospital of Inner Mongolia Medical University (NO. KY 2022017). All procedures were carried out in line with the relevant guidelines and regulations. All patients signed the informed consent according to institutional guidelines.

Specimen collection and DNA extraction

DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) was used for DNA extraction of Transbronchial lung biopsy, malignant pleural effusion and fresh surgical tissue. QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) was used for DNA extraction of 136 FFPE samples. The extraction was conducted according to the instructions of the Kit manufacturer. Then, 10 mL blood sample was collected and centrifuged at 1600 g for 15 min to isolate the plasma. Cell free DNA (cfDNA) was extracted from the isolated plasma with the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) followed the manufacturer's instructions.

Detection of EGFR mutations with Anlongen LAN-ARMS EGFR mutation detection kit

EGFR mutations were detected with Anlongen LAN-ARMS *EGFR* Mutation Detection Kits. This Detection Kit covers 25 most commonly *EGFR* mutations (Table S1). Briefly, the PCR reaction mixture included 21 μL mixture, 2 μL Taq DNA polymerases and 2 μL DNA templates. The PCR reactions were performed on ABI 7500 PCR. The reaction conditions were: 1 cycle at 50°C for 2 min, 95°C for 5 min for incubation, then 40 cycles at 95°C for 10 s, and 60°C for 45 s.

Detection of gene mutations with multiplex PCR-Based NGS

Tissue samples were subjected to DNA cleavage with a double stranded DNA Fragmentase (Roche Sequencing and Life Science, Indianapolis, IL 46250, USA). Multiplex PCR primers were designed for each variant. KAPA Library Preparation Kit (KAPA Biosystems, Wilmington, MA 01887, USA) was used to construct the DNA library. The constructed DNA library was used for future sequencing on life proton (BioelectronSeq 4,000). The raw data were filtered through fastq, and the raw data were ≥88%, Q20 ≥80%, target region coverage ≥99.8%, average sequencing depth in tissues ≥1500×. The somatic variants of the *EGFR* were called as following: the read depth ≥7.5×, the variant allele frequency (VAF) for DNA ≥1%.

Statistical analysis

The sensitivity, specificity, PPV, NPV and concordance rate of the plasma *EGFR* mutation detection Kit were analyzed, and the tumor tissue results were the standard reference in this study. SPSS 20.0 was used for significance analysis of data, and $P<0.05$ was defined as a significant difference. The Chi-square test was used for testing the clinical characteristics and EGFR mutation. The ORR after *EGFR*-TKIs treatment was evaluated according to Response Evaluation Criteria in Solid Tumors, version 1.1. Sigmaplot 10.0 software was used in this paper.

Results

Association between EGFR mutations and clinical characteristics

Patient enrollment and sample collection of the patient subgroups were displayed in **Figure 1**. **Table 1** displayed the clinical characteristics of these patients. Of the 179 patients, 129 patients were over sixty years old, 91 patients were male, 145 patients were classified as stage IV, and 123 patients were never smokers.

We analyzed the correlation between *EGFR* mutations and patient characteristics (**Table 1**). Sex was significantly associated with tissue *EGFR* mutations, but was not significantly associated with plasma *EGFR* mutations. Smoking history was significantly associated with both tissue and plasma *EGFR* mutations. There was no significant correlation between other clinical characteristics and *EGFR* mutations.

EGFR mutation analysis between tissue and plasma

Tumor drive gene was detected in tissue samples but also in liquid biopsy samples, such as plasma, pleural effusion supernatant and cerebrospinal fluid. Therefore, *EGFR* mutations were detected in both tissue and tumor associated plasma cfDNA. The detection of gene mutations in plasma has also been recommended before EGFR-TKI targeted therapy for the NSCLC patients.

Of the 111 results of the tissue samples detected by NGS, the detection rate of the L858R, L861Q, 19DEL, G719X, L858R, T790M, L858R, L858M, T790M, 19DEL and 19DEL Met14 was 16.22% (18/111), 2.70% (3/111), 17.12%

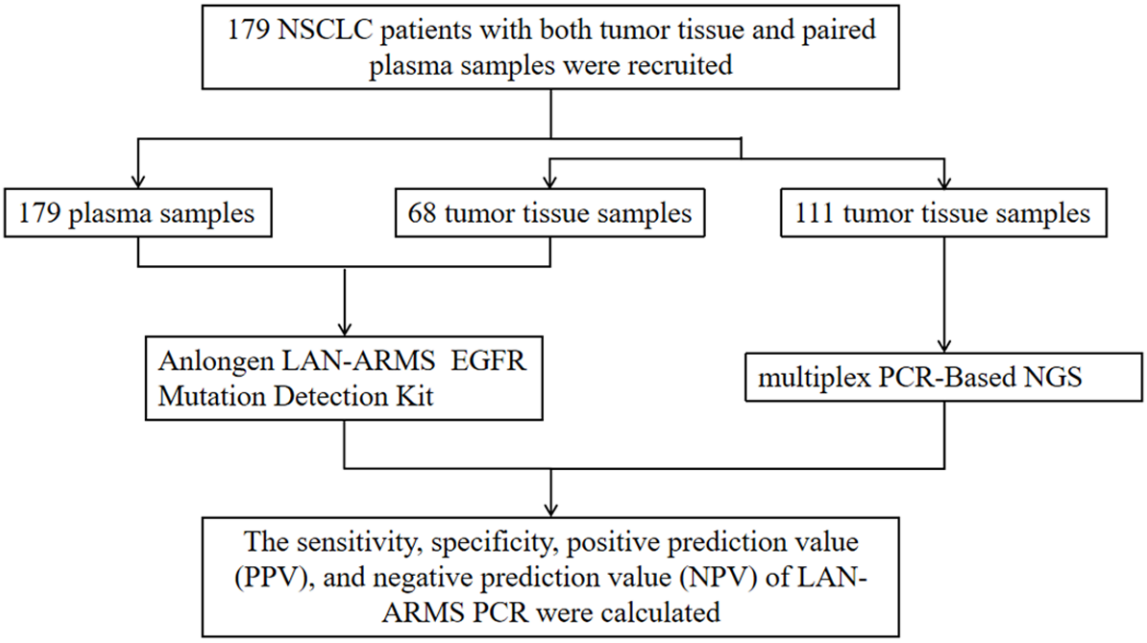

Figure 1. Patient enrollment and sample collection of the patient subgroups. NSCLC, Non-small-cell lung cancer; EGFR, Epidermal growth factor receptor; NGS, Next-generation sequencing; PPV, Prediction value; NPV, Negative prediction value; LAN-ARMS, Locked nucleic acid-amplification refractory mutation system.

Table 1. Clinical characteristics of 179 patients with lung cancer

Characteristic	Tissue (n=179)			Plasma (n=179)		
	Mut	Wt	P	Mut	Wt	P
Age (years)			0.501			0.116
<60 (N=50)	24	26		16	34	
≥60 (N=129)	53	76		26	103	
Sex			0.000			0.158
Male (N=91)	27	64		17	74	
Female (N=88)	50	38		25	63	
Stage			0.163			0.501
IIIB (N=34)	11	23		6	28	
IV (N=145)	66	79		36	109	
Smoking history			0.008			0.007
Never smokers (N=123)	61	62		36	87	
Current or former (N=56)	16	40		6	50	

Note: Mut, Mutation; Wt, Wild type.

(19/111), 0.90% (1/111), 1.80% (2/111), 0.90% (1/111), 0.90% (1/111) and 0.90% (1/111), respectively. The other 68 tissue samples were detected by ARMS. The detection rate of the L858R, L861Q, 19DEL, G719X, L858R T790M, L858R L858M, and T790M 19DEL was 20.59% (14/68), 4.41% (3/68), 19.12% (13/68), 1.47% (1/68), 0.00% (0/68), 0.00% (0/68), and 0.00% (0/68), respectively (**Figure 2**).

For plasma samples, *EGFR* mutations were detected in 42 of the 179 patients. The type of the mutation included L861Q, L858R, 19DEL, G719X, T790M and L858R+T790M. Of the 111 samples of the plasma, the detection rate of the L858R, L861Q, 19DEL, G719X, L858R T790M, L858R L858M, T790M 19DEL and T790M was 11.71% (13/111), 0.00% (0/111), 9.00% (10/111), 0.90% (1/111), 0.90% (1/111), 0.00% (0/111), 0.00% (0/111) and 0.00% (0/111), respectively. The other 68 plasma samples showed that the detection rate of the L858R, L861Q, 19DEL, G719X, L858R T790M, L858R L858M, T790M 19DEL and T790M was 13.24% (9/111), 1.47% (1/111), 10.29% (7/111), 0.00% (0/111), 0.00% (0/111), 0.00% (0/111), 0.00% (0/111) and 0.00% (0/111), respectively (**Figure 2**).

The summary of the *EGFR* mutations of the tissue and plasma samples were shown in

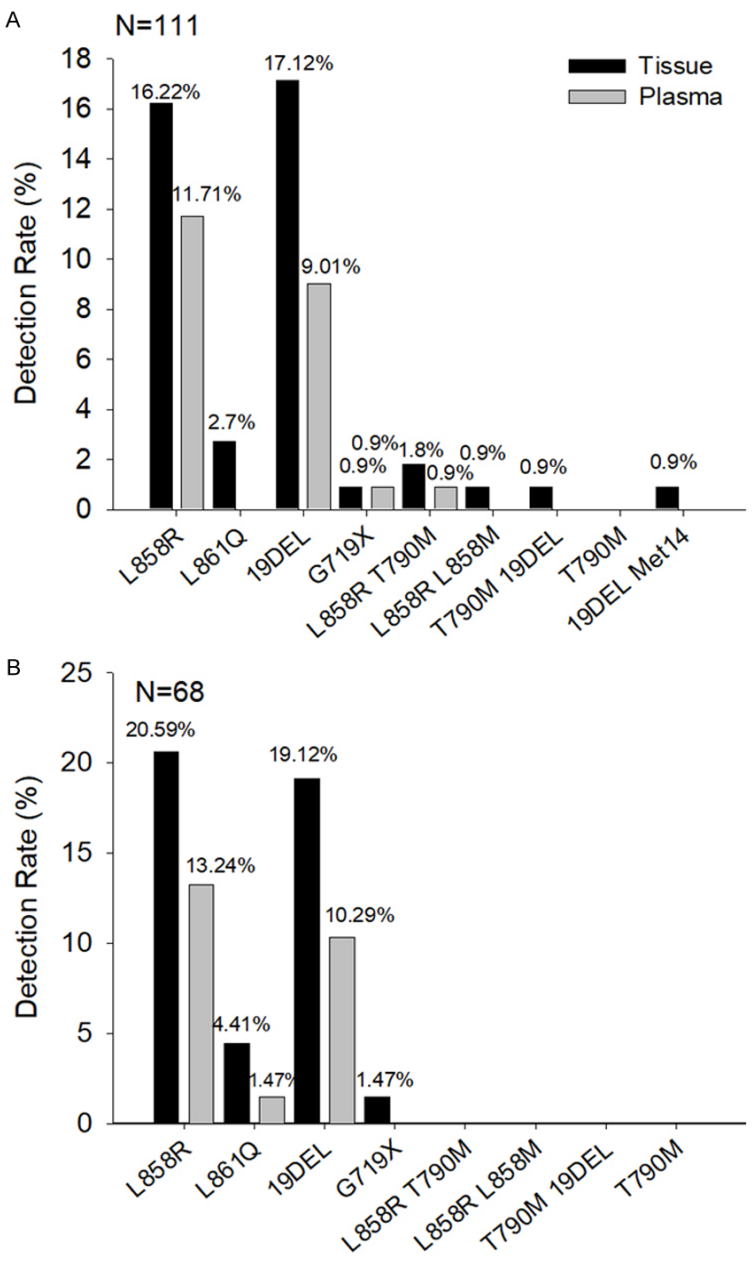

Figure 2. EGFR mutations in tumor tissue and plasma samples. A. EGFR mutations of 111 tissue samples detected by multiplex PCR-Based NGS sequence; B. *EGFR* mutations of 68 tissue samples detected by LAN-ARMS. EGFR, Epidermal growth factor receptor; NGS, Next-generation sequencing; LAN-ARMS, Locked nucleic acid-amplification refractory mutation system.

Table 2. Compared with tissue results, the sensitivity and specificity of LAN-ARMS in detecting *EGFR* mutations were 57.18% and 98.04%, respectively. The PPV was 95.24%, and NPV was 72.99%. The overall agreement of *EGFR* mutation detection was 78.21% (140/179).

In the 179 paired samples, the *EGFR* mutation results were inconsistent in 40 pairs of tissue and plasma samples. Of these 40 inconsistent results, sample ID 165 with L858R T790M mutation in tissue and L858R mutation in plasma was also listed in **Table 3**. Tissue and plasma results of sample ID 165 were all defined as mutation. Therefore, they were all classified as mutation consistent group in **Table 2**. Among the other 39 paired samples, *EGFR* mutations of were detected positive in 14 tissue samples by LAN-ARMS PCR, and the paired 14 plasma samples were detected negative by LAN-ARMS PCR. Of the remaining 25 tissue cases detected by NGS, 22 cases were Wt in plasma, and 3 cases had different mutations. Two cases (patient ID 63 and 178) were Wt in tissue, whose *EGFR* mutations were L858R, G719X in plasma.

EGFR mutations for predicting ORR of EGFR-TKIs

In tumor tissues, there were a total of 76 patients with *EGFR* mutations, and 41 of them received Gefitinib or Icotinib treatment as first-line EGFR-TKIs therapy. Twenty-eight (68.29%, 28/41, 95% CI, 53.4-83.2) patients achieved a partial response (PR) or complete response (CR). Thirty patients with tissue and plasma *EGFR* mutations received EGFR-TKIs treatment, and 22 of them achieved a PR or CR. Therefore, the ORR was 73.33% (95% CI, 56.5-90.1). There were no significant differences among the three subgroups (P=0.794; **Figure 3**).

Discussion

Since the apoptosis of the tumor cell results in cfDNA releasing into the plasma, tumor

Table 2. The sensitivity, specificity and consistency of Anlongen LAN-ARMS EGFR Mutation Detection Kit for EGFR mutation detection in plasma

Parameter	Mut	Wt	Total	Sensitivity	Specificity	PPV	NPV	Overall agreement
plasma								
Mut	40	2	42	57.18% (40/77)	98.04% (100/102)	95.24% (40/42)	72.99% (100/137)	78.21% (140/179)
Wt	37	100	137					
Total	77	102	179					

Note: LAN-ARMS, Locked nucleic acid-amplification refractory mutation system; EGFR, Epidermal growth factor receptor; PPV, Prediction value; NPV, Negative prediction value; Mut, Mutation; Wt, Wild type.

derived gene can be detected not only in tumor tissue sample, but also in liquid biopsy sample, such as plasma, pleural effusion supernatant, cerebrospinal fluid and so on. The gene mutations in plasma cfDNA could not only be used for guiding targeted therapy, but also for predicting survival in tumor patients [24]. *EGFR* mutation detection based on ARMS PCR has been used successfully in clinical practice. The use of ARMS *EGFR* mutation detection Kit (Amoy Diagnostics, Xiamen, China) has been approved for advanced lung cancer patients by Chinese National Medical Products Administration (NMPA). Cobas-ARMS (cobas® EGFR Mutation Test v2) has been approved by the U. S. Food and Drug Administration (FDA) for *EGFR* mutation detection in plasma. In this study, we detected the *EGFR* mutations in the plasma with Anlongen LAN-ARMS *EGFR* Mutation Detection Kits. The sensitivity, specificity, PPV and NPV of the Kit were analyzed and compared with the results of tissue detection.

Of the 179 patients, *EGFR* mutations of 111 tissue samples were detected by NGS sequence, and the remaining 68 tissue samples were detected by LAN-ARMS. The detection rate of the L858R, L861Q, 19DEL and G719X in tissue samples by NGS sequence was lower than that in tissue samples by LAN-ARMS, which is not consistent with other results. This maybe result from small number of tissue samples for LAN-ARMS detection. The tissue samples had higher *EGFR* mutation rate compared with plasma samples, both NGS vs. LAN-ARMS and LAN-ARMS vs. LAN-ARMS. Plasma cfDNA has been recommended for alternative detection when tissue was not available. A meta-analysis of plasma cfDNA for *EGFR* mutation detection showed that the sensitivity was ranged from 48.2% to 67.4%, and the specificity was ranged from 93.5% to 100% [15, 21, 22, 25].

In this study, tissue detection result was the gold standard, and the sensitivity, specificity, PPV and NPV of the plasma detection was 52.63%, 98.06%, 95.24% and 73.72%, respectively. It has been reported that the sensitivity, specificity, PPV and NPV of the ADx-ARMS Plasma Detection Kit was 52.90%, 98.80%, 98.40% and 59.70%, respectively [26]. The sensitivity, specificity and PPV of this study was similar with that of ADx-ARMS for *EGFR* mutation detection. The NPV of this LAN-ARMS PCR was higher than that of ADx-ARMS Plasma Detection Kit. The overall detection agreement of this study was 78.77% (141/179), which is also higher than that of ADx-ARMS Plasma Detection Kit. Consistent with previous study, ARMS PCR in plasma was highly predictive of identical mutations of tissue [25, 27].

Between tissue and plasma samples, only 40 cases showed inconsistent *EGFR* mutations in this study. *EGFR* of patient ID 63 and 178 were Wt in tissue detected by NGS sequence, but *EGFR* mutations of plasma were detected by LAN-ARMS PCR. The reason for these inconsistent maybe the heterogeneity of the tissue [28, 29]. In order to overcome the missed detection due to tumor heterogeneity in the clinic, tissue detection combined with plasma cfDNA detection might be beneficial. The tissue results of sample ID 165 was L858R-T790M double mutations, whereas the mutations of plasma were L858R, which means T790M mutation was not detected in the plasma. This may be due to the double mutation detection rate of LAN-ARMS PCR was lower than that of NGS. T790M as a resistant mutation was only detected in tissue. It is suggested that these patients may lose the opportunity to receive drug-resistant targeted therapy. Therefore, it is necessary to detect *EGFR* mutations in both tissue and plasma before the clinical treatment.

EGFR mutations as well as the treatment response to EGFR-TKIs have been reported previously [15, 19, 30, 31]. It has been reported th-

Table 3. The list of inconsistent mutations of EGFR between tissue and plasma samples

Sample ID	Tissue		plasma	
	ARMS	NGS	ARMS	NGS
14	G719X		Wt	
15	19DEL		Wt	
20	L858R		Wt	
27	19DEL		Wt	
29	19DEL		Wt	
36	19DEL		Wt	
42	L858R		Wt	/
46	L858R		Wt	
47		L861Q	Wt	
54		L858R	Wt	
63		Wt	L858R	
64		L858R, L858M	Wt	
66		19DEL	Wt	
67		19DEL	Wt	
75		L858R	Wt	
79		T790M, 19DEL	Wt	
80		L861Q	Wt	
88		G719A	Wt	
89		19DEL	Wt	
93	L858R		Wt	
105		L858R	Wt	
109	19DEL		Wt	
110		19DEL	Wt	
112	L858R		Wt	
118	L861Q		Wt	
119		L861Q	Wt	
125	L861Q		Wt	
127		19DEL	Wt	
131		L858R	Wt	
139		19DEL	Wt	
144		19DEL	Wt	
146		L858R	Wt	
148		19DEL	Wt	
159		L858R	Wt	
165		L858R, T790M	L858R	
167		L858R	Wt	
173	19DEL		Wt	
176		19DEL	Wt	
178		Wt	G719X	
179		19DEL	Wt	

Note: Represent not detected by ARMS/NGS. EGFR, Epidermal growth factor receptor; ARMS, Amplification refractory mutation system; Wt, Wild type.

at patients with plasma *EGFR* mutations had higher ORR as compared with those with plasma *EGFR* Wild type [8, 15]. In this study, patients with both tissue and plasma *EGFR* mutations had higher ORR compared with those with only tissue *EGFR* mutations. It was suggested that detection of plasma *EGFR* mutations was necessary for selecting patients who are able to benefit more from EGFR-TKIs treatment.

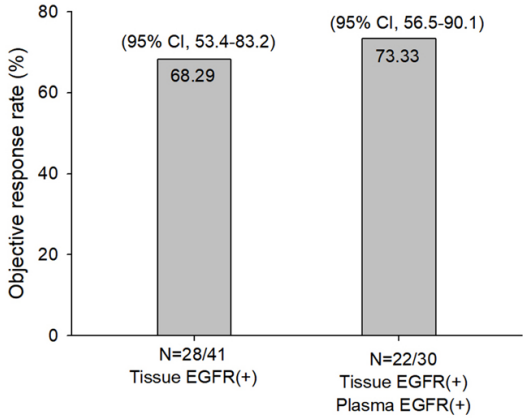

Figure 3. Objective response rate analysis in patients who were tumor tissue EGFR mutation positive, and both tumor tissue and plasma EGFR mutation positive. EGFR, Epidermal growth factor receptor.

In conclusion, in tissue and plasma samples, Anlongen LAN-ARMS *EGFR* Mutation Detection Kits had a high sensitivity and specificity for *EGFR* detection. Anlongen LAN-ARMS *EGFR* Mutation Detection Kits had the advantages of easy-to-operate and high sensitivity. It is suggested that tissue detection combined with plasma detection can improve the mutation rate. Therefore, the combined detection of the tumor tissue and plasma should be suggested in clinical practice so as to improve the clinical benefit of patients.

Acknowledgements

We thank Anhui Anlongen technology co., Ltd for gene mutation detection of the samples. Recruitment was funded by Special fund for clinical research of Wu Jieping Medical Foundation in 2018 (Project Number: 320.6750.17570), and 2019 Beijing Medical and Health Foundation (funded by the medical science research foundation, Project Number: B182827). The funding bodies had no role in: the design of the study; data collection, analysis, or interpretation; or, in writing the manuscript.

This study was approved by the Human Research Ethical Committee of the Affiliated Hospital of Inner Mongolia Medical University. Written informed consent was obtained from study participants according to institutional guidelines.

Disclosure of conflict of interest

None.

Abbreviations

EGFR, Epidermal growth factor receptor; NSCLC, Non-small-cell lung cancer; ctDN, Acirculating tumor DNA; LAN-ARMS, Locked nucleic acid-amplification refractory mutation system; PPV, Prediction value; NPV, Negative prediction value; ddPCR, Droplet digital PCR; NGS, Next-generation sequencing.

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Table S1. The site information of Anlongen EGFR Mutation Detection Kit

Number	Exon	Nucleotide	Amino acid
1	18	2156G>C	G719A
2	18	2155G>A	G719S
3	18	2155G>T	G719C
4	19	2235_2249 del 15	E746_A750del (1)
5	19	2236_2250 del 15	E746_A750del (2)
6	19	2236_2253 del 18	E746_T751del
7	19	2237_2251 del 15	E746_T751>A
8	19	2237_2254 del 18	E746_S752>A
9	19	2238_2255 del 18	E746_S752>D
10	19	2239_2247 del 9	L747_E749del
11	19	2239_2253 del 15	L747_T751del
12	19	2239_2256 del 18	L747_S752del
13	19	2240_2251 del 12	L747_T751>S
14	19	2240_2254 del 15	L747_T751del
15	19	2240_2257 del 18	L747_P753>S
16	19	2235_2252>AAT (complex)	E746_T751>I
17	19	2237_2250>T (complex)	E746_S752>V
18	19	2238_2248>GC (complex)	L747_A750>P
19	19	2238_2252>GCA (complex)	L747_T751>Q
20	19	2239_2248 TTAAGAGAAG>C (complex)	L747_A750>P
21	19	2239_2251>C (complex)	L747_T751>P
22	19	2239_2258>CA (complex)	L747_P753>Q
23	20	2369C>T	T790M
24	21	2573T>G	L858R
25	21	2582T>A	L861Q