

Detection of epidermal growth factor receptor mutation in plasma as a biomarker in Chinese patients with early-stage non-small cell lung cancer

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Purpose: This preplanned exploratory analysis was conducted to reveal the true status of correlation between tissue and plasma detection for early-stage non-small cell lung cancer (NSCLC) epidermal growth factor receptor (EGFR) mutations, knowing that specific subgroups of NSCLC patients may be potential candidates for EGFR mutation analysis by using plasma samples.

Materials and methods: Tissue samples were surgically resected from 198 patients with stage I–IV NSCLC, where stage I_A to III_A accounted for 92.4%. EGFR mutations in all these tissues were positive. Paired plasma EGFR mutations were detected by real-time polymerase chain reaction; concentration of cell-free DNA (cfDNA) in plasma was measured by ultraviolet spectrophotometry.

Results: EGFR-activating mutation was detected in 34 plasma samples, and their mutation types were matched with that in tissue. The sensitivity of EGFR mutation for the 198 paired tissue and plasma samples was 17.2%. The sensitivity positively correlated with disease stage and negatively correlated with tumor differentiation. The sensitivity of stage I_A, I_B, II_A, II_B, and III_A was 1.6%, 7.9%, 11.1%, 20%, and 33.3%, respectively; the sensitivity of high differentiation was 0% versus 36.8% for poor differentiation. There was no correlation between plasma cfDNA concentration and patient characteristics.

Conclusion: We recommend using plasma cfDNA as a biomarker in stage III_A or poorly differentiated tumors for gene diagnosis, especially in patients whose tissue samples cannot be obtained by surgery. Plasma samples can really reflect the patients' EGFR mutation types and may contain comprehensive genotypic information that comes from different parts of the tumor than tissue specimens. The concentration of plasma cfDNA does not vary with patient characteristics.

Keywords: EGFR mutations, NSCLC, plasma, early stage, ARMS, targeted therapy

Introduction

Lung cancer is the most common cause of cancer-related death in both men and women worldwide,¹ and 80%–90% of all lung cancers are non-small cell lung cancer (NSCLC).² Currently, patients with NSCLC are generally treated with curative intent using surgery, chemotherapy, and radiation therapy, or a combined modality approach. However, the 5-year relative survival rate for NSCLC is only approximately 15%,³ although this status is being changed by molecular targeting treatment. Tyrosine kinase inhibitors (TKIs) are representative molecular targeting drugs. Epidermal growth factor receptor (EGFR)-activating mutations have significant impact on the clinical treatment of NSCLC patients using EGFR-TKIs.^{4–6} The Iressa Pan-Asia Study showed that objective response rate % and progression-free survival (PFS) of EGFR-TKI therapy reach up to 71.2%

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and 9.8 months, respectively, which is significantly higher than chemotherapy, whose objective response rate % and PFS is only 47.3% and 6.4 months, respectively.⁷ Several studies well demonstrated that PFS was increased significantly in TKI-treated patients with EGFR-positive NSCLC.^{8–10}

EGFR mutation detection using tissue samples from NSCLC patients is regarded as the gold standard in the prediction of TKI treatment responses and prognoses. However, it is sometimes difficult to collect tumor tissues for biomarker analysis,¹¹ and therefore great interest in using surrogate samples such as serum and plasma samples has been aroused. The biological mechanism underlying the increased release of cell-free DNA (cfDNA) remains to be fully understood. Several reviews detailed the biology and mechanism of DNA release into circulation.^{12–14} Elements of apoptosis and necrosis, especially of large tumors, and the possibility of active release of cfDNA from tumors have been reported.¹⁵ The detection of EGFR mutations in plasma may provide a noninvasive and surrogate source of genotypic information that might provide dynamic monitoring to facilitate clinical decision-making at the time of diagnosis and in the later course of disease, especially in TKI-treated patients. Recently, Mok et al¹⁶ have confirmed that dynamic changes in the cfDNA EGFR mutation status relative to the baseline may predict clinical outcomes of patients using EGFR-TKIs.¹⁶

Although a series of previous studies have shown that the use of plasma to detect EGFR mutations is feasible in the advanced stage of the disease, the reported sensitivity varied considerably. Brevet et al¹⁷ showed that the sensitivity and specificity, respectively, were 44% and 85% in stage III–IV NSCLC versus 100% and 89% in stage III_B–IV, as reported by He et al.¹⁸ However, studies about plasma EGFR mutation detection in early-stage NSCLC are rarely reported. Zhao et al assessed plasma EGFR mutations in stage I–IV patients by mutation-enriched polymerase chain reaction (PCR) and reported an overall sensitivity of 36% versus 45.9% for stage I_A–III_A.¹⁹ On the contrary, Ren et al²⁰ showed that the sensitivity was 0 in stage I_A–III_A patients using direct sequencing, indicating that direct sequencing is not sensitive enough to detect a very low level of EGFR mutation in plasma of early stage. So, both of the two studies were vulnerable in assessing plasma EGFR mutations in the early-stage NSCLC patients.

The frequently used methods for EGFR mutation detection in plasma include direct sequencing, Scorpion-amplification-refractory mutation system (ARMS), denaturing high-performance liquid chromatography, multiplex PCR, and digital droplet PCR. The Scorpion-ARMS method is

highly sensitive and fast for detection of known mutations.²¹ In addition, it is easy and simple to operate. Goto et al²² reported a sensitivity of 43.1% and a specificity of 100% with the Scorpion-ARMS method in a Japanese subgroup of patients from the Iressa Pan-ASia Study. A recent first-line, single-arm study on gefitinib reported a concordance rate of 94.3%, sensitivity of 65.7%, and specificity of 99.8% using the Scorpion-ARMS method.²³ However, the aforementioned studies used different detection methods and therefore lacked standardization. It is notable that few studies have reported on early-stage (I_A–III_A) patients and the correlation between sensitivity and patient characteristics.

In the present study, we used the Scorpion-ARMS method to explore the mutation status in the plasma of NSCLC patients whose tissue EGFR mutations were positive, in an attempt to provide credible experimental data for clinical screening of specific subgroups of early-stage patients who may be the best candidates for EGFR mutation analysis using blood cfDNA for diagnosis and dynamic monitoring of cfDNA and prediction of TKI resistance. In addition, we also attempted to use plasma EGFR mutation detection to assess the prognosis of early-stage NSCLC patients.

Materials and methods

Patients and materials

Included in this study were 198 NSCLC patients who had not received radiotherapy, chemotherapy, or targeted therapy previously and had been diagnosed and treated at the Department of Thoracic Surgery of Tangdu Hospital of the Fourth Military Medical University (Xi'an, People's Republic of China) between February 2014 and June 2015. Our study was approved by the Review Board of the said university. Written informed consent was obtained from all patients before any study-related procedure, including the provision of samples for biomarker testing.

Tissue samples

All tissue samples were obtained by various operations in the Department of Thoracic Surgery of Tangdu Hospital. All tumor samples were evaluated histologically by pathologists from Tangdu Hospital to confirm the NSCLC diagnosis. A part of stage IV patients who had metastasis were treated by surgical resection, aiming to obtain tumor by surgery for pathological diagnosis. According to manufacturer's protocol, DNA was extracted from fresh tumor samples using an Amoy Tissue DNA Extraction Kit (Amoy Diagnostics Corporation, Xiamen, People's Republic of China). Then Human EGFR Mutation Detection Kit (Amoy Diagnostics Corporation) and real-time PCR (Agilent StrataGene

Mx3000P, Palo Alto, CA, USA) was used for EGFR mutation detection (including G719X in exon 18, deletions mutations in exon 19, T790M mutation in exon 20, exon 20 insertions, and L858R in exon 21, L861Q and S768I). All tissue EGFR detections of the 198 NSCLC patients were positive.

Plasma samples

Blood samples were collected 1 day before the operation and prepared in an anticoagulant-treated (ethylenediaminetetraacetic acid) tube for analysis within 4 hours after sampling.²⁴ For each patient, a 5 mL blood sample was needed; it was centrifuged (Sigma Laborzentrifugen 3–30K, Osterode am Harz, Germany) at $825\times g$ for 15 minutes and then the 2 mL supernatant, or plasma, was moved in a sterile tube and stored at -80°C until use. Paired plasma samples were collected from the 198 patients whose tissue mutations were positive.

cfDNA extraction

cfDNA was extracted from 2 mL plasma using an Amoy plasma/serum DNA Extraction Kit (Amoy Diagnostics Corporation) according to manufacturer's protocol. The concentration and purity of the extracted DNA were determined using a spectrophotometer (DU800 UV/VIS Spectrophotometer, Beckman Coulter, Pasadena, CA USA). The extracted DNA was stored at -20°C until use.

Real-time PCR analysis for EGFR mutation

The Scorpion-ARMS method is highly sensitive and fast,²¹ and can be validated to detect a mutant as low as 1.0%.²⁵ cfDNA (45 μL) was used for EGFR mutation detection using a Human EGFR Mutation Detection Kit (Amoy Diagnostics Corporation) by real-time PCR (Agilent StrataGene Mx3000P) according to manufacturer's protocol. The PCR reaction included three stages: first stage, 1 cycle at 95°C for 5 minutes; second stage, 15 cycles at 95°C for 25 seconds, 64°C for 20 seconds, 72°C for 20 seconds; third stage, 31 cycles at 93°C for 25 seconds, 60°C for 35 seconds, 72°C for 20 seconds. The real-time PCR result of plasma mutation was read and analyzed by two professional investigators. Any disagreement between the two investigators was resolved by discussion with a third observer. Experimental data were obtained and recorded independently by the investigators who were blinded to the clinical data until statistical analysis.

Statistical analysis

SPSS software (version 22.0, IBM Software, Armonk, NY, USA) was used to analyze the data. The chi-square test or

Fisher's exact test was used to assess the relationship between the presence of EGFR mutations in plasma with NSCLC and patient characteristics. One-way analysis of variance was used to assess the relationship between the concentration of cfDNA and patients characteristics. A *P*-value of less than 0.05 was considered to be statistically significant.

Results

Patient characteristics and the results of subgroup sensitivity analysis

A total of 198 patients were enrolled into our study between February 2014 and June 2015 at the Department of Thoracic Surgery of Tangdu Hospital. The patients were composed of 86 men and 112 women ranging in age from 36 to 78 years. The initial clinical records of the 198 patients were reviewed by the investigators, showing 52 smokers and 146 never-smokers. A person who smoked more than 100 cigarettes in his/her past history was defined as a smoker. Of the 198 patients, 183 had lung adenocarcinoma, eight had squamous cell carcinoma, four had adenosquamous carcinoma, and three had other types of carcinoma, including large cell carcinoma, sarcomatoid carcinoma, and bronchoalveolar carcinoma. Disease stages were classified according to the National Comprehensive Cancer Network guideline in 2014. The percentage of patients with stage I_A to III_A disease was 92.4%, including 62 stage I_A, 38 stage I_B, 18 stage II_A, five stage II_B, and 60 stage III_A NSCLC. The remaining were three stage III_B cases and 12 stage IV cases. Of the 198 patients, 45 were well differentiated, 108 were moderately differentiated, 38 were poorly differentiated, and seven were uncertain.

In total, 34 plasma samples were EGFR mutation positive. The result of analysis on the correlation between the patient characteristics and the sensitivity of plasma detection showed no significant difference between the ratio and age, sex, smoking history, and histological type. The overall sensitivity of the EGFR mutation status in our study was 17.2%. However, the sensitivity was enormously different in tumor, node, and metastasis (TNM) stages and differentiation subgroups. The sensitivity of I_A and I_B was 1.6% and 7.9%, respectively, which was significantly lower than 33.3% in III_A. For patients with poor differentiation, the sensitivity was 36.8%, which was significantly higher than that in patients with high differentiation (0%, *P*=0.00) and moderate differentiation (15.7%, *P*=0.01). Table 1 shows patient characteristics and the results of subgroup sensitivity analysis. Figure 1 shows the tendency of sensitivity in subgroups of differentiation and TNM stage.

Table I Patient characteristics and the results of subgroup sensitivity analysis

Patients	N (%)	Sensitivity (%)	Plasma EGFR		P-value
			+	–	
Sex					0.502
Male	86 (43.4)	15.1	13	73	
Female	112 (56.6)	18.8	21	91	
Age, years					0.238
≥60	88 (44.4)	13.6	12	76	
<60	110 (55.6)	20	22	88	
Smoke					0.409
Ever ^a	52 (26.3)	13.5	7	45	
Never	146 (73.7)	18.5	27	119	
Histopathology type					0.069
Squamous cell	8 (4.04)	0	0	8	
Adenocarcinoma	183 (92.42)	16.9	31	152	
Adenosquamous carcinoma	4 (2.02)	25	1	3	
Other types ^b	3 (1.52)	66.7	2	1	
Differentiation					0.000
High	45 (22.7)	0	0	45	
Moderate	108 (54.6)	15.7	17	91	
Poor	38 (19.2)	36.8	14	24	
Uncertain	7 (3.5)	42.9	3	4	
Disease stage					0.000
I _A	62 (31.3)	1.6	1	61	
I _B	38 (19.2)	7.9	3	35	
II _A	18 (9.1)	11.1	2	16	
II _B	5 (2.5)	20	1	4	
III _A	60 (30.3)	33.3	20	40	
III _B	3 (1.5)	33.3	1	2	
IV	12 (6.1)	50	6	6	
Total	198 (100)	17.2	34	164	

Notes: ^aA person who smoked more than 100 cigarettes in his/her past history was defined as an ever smoker. ^bOther types included large cell, sarcomatoid, and bronchoalveolar carcinoma.

Abbreviation: EGFR, epidermal growth factor receptor.

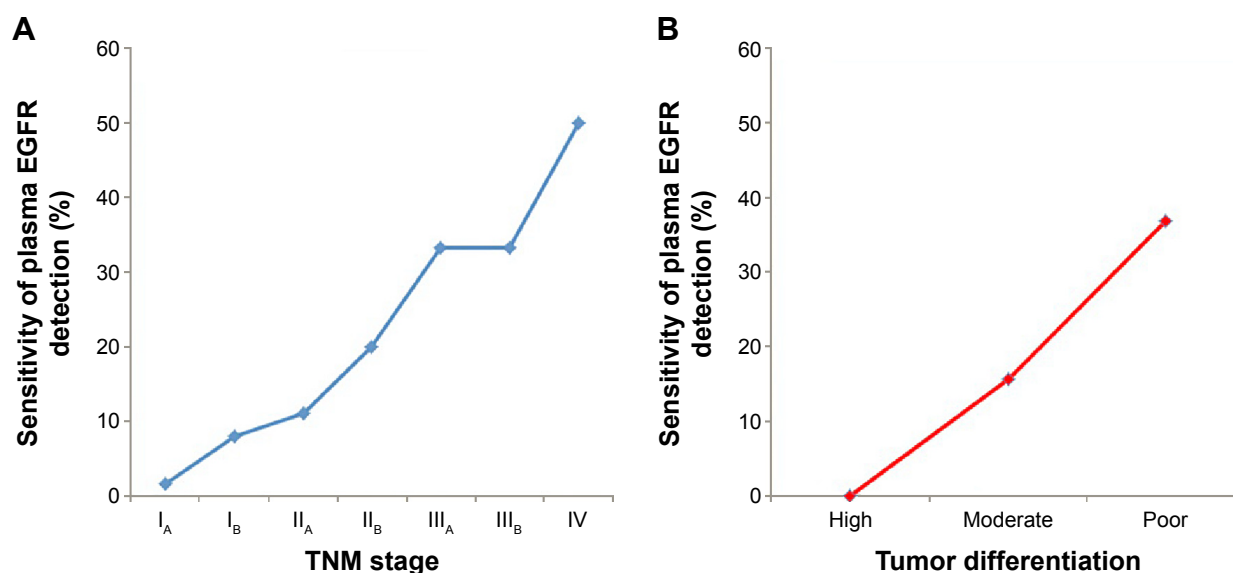


Figure 1 The sensitivity tendency of disease stage and tumor differentiation.

Notes: The sensitivity tendency of disease stage is shown by a blue line in graph (A), and the sensitivity tendency of tumor differentiation is shown by a red line in graph (B). X-axes are the TNM stage and tumor differentiation, respectively. Y-axes are sensitivity of plasma EGFR detection. Obviously, the sensitivity was positively correlated with disease stage and negatively correlated with tumor differentiation.

Abbreviations: EGFR, epidermal growth factor receptor; TNM, tumor, node, and metastasis.

Plasma and tissue mutation types

Tissue and plasma mutations were detected using the Scorpion-ARMS method, showing that most mutations were single mutations and the others were double mutations.

The details of plasma and tissue single mutations and the positive ratio of different single mutation types are summarized in Table 2. There were 188 tissue single mutations and 32 plasmas single mutations. Exon 19 deletion mutation (19-del) and exon 21 L858R point mutation (L858R) were more frequently seen in the tissue samples, which is consistent with our previous study.²⁶ In the plasma samples, 23 exon 19 deletion mutations, five exon 21 L858R mutations, three L861Q mutations, and one exon 20 insertion were detected, without seeing T790M, G719X, and S768I mutations. The positive mutation type in the plasma sample was identical to that in the paired tissue sample. The result indicated a high consistency between the positive mutation types detected in the plasma and corresponding tissue.

The positive ratio of exon 19 deletion mutations was 22.5%; the positive ratio of L858R, 20 insertions, and L861Q mutation was 7.0%, 14.3%, and 75%, respectively. However, T790M, G719X, and S768I were all zero. Our study showed that the positive ratio of mutation types in plasma was statistically different from each other ($P=0.003$). We further analyzed the correlation between the positive ratio of 19 deletions and L858R, and found that the positive ratio of 19 deletions was significantly better than that of L858R in plasma ($P=0.011$).

Double mutations are shown in Table 3. Among the ten tissue samples with double mutations, there were G719X and S768I mutations in four, 19 deletions and L858R mutations in two, and L858R mutations and T790M in two samples. The other two tissue samples were detected with 19 deletions, G719X and 19 deletions, T790M, respectively. However, both of their paired plasma samples were mutation negative. Noticeably, the mutation type of the two plasma samples

Table 2 The positive ratios of single mutation types

Mutation type	n (%)	Plasma mutation		Tissue mutation
		+	–	
19 Del	22.5	23	79	102
L858R	7.0	5	66	71
L861Q	75	3	1	4
20-Ins	14.3	1	6	7
T790M	0	0	0	0
G719X	0	0	3	3
S768I	0	0	1	1
Total	17.0	32	156	188

Abbreviations: 19 Del, exon 19 deletion mutations; 20-Ins, exon 20 insertion mutations; G719X, exon 18 G719A, G719S, G719C mutations; L858R, exon 21 L858R point mutation; L861Q, exon 21 L858R point mutation; T790M, exon 20 T790M point mutation; S768I, exon 20 S768I point mutation.

Table 3 The details of plasma and tissue double mutations

Plasma	Paired tissue	N
19 Del+L858R	L858R	1
19 Del+L861Q	L861Q	1
–	19 Del+L858R	2
–	19 Del+G719X	1
–	19 Del+T790M	1
–	L858R+T790M	2
–	G719X+S768I	4

was basically the same as their paired tissue samples, except additional exon 19 deletion mutation, which was not detected in the paired tissue samples.

Correlation between the concentration of cfDNA and patient characteristics

Table 4 shows the concentration of extracted cfDNA in a 2 mL plasma sample. The median concentration was 17.34 µg/mL

Table 4 The concentration of cell-free DNA and patient characteristics

Patients	Median concentration (µg/mL)	P-value
Sex		0.889
Male	17.49	
Female	17.23	
Age, years		0.384
≥60	18.25	
<60	16.62	
Smoke		0.930
Ever ^a	17.29	
Never	17.49	
Histopathology type		0.516
Squamous cell	19.83	
Adenocarcinoma	17.47	
Adenosquamous carcinoma	13.62	
Other types ^b	8.26	
Differentiation		0.170
High	18.61	
Moderate	16.21	
Poor	20.20	
Uncertain	11.33	
Disease stage		0.264
I _A	16.72	
I _B	20.86	
II _A	18.17	
II _B	25.69	
III _A	15.55	
III _B	15.70	
IV	14.07	
Plasma mutation status		0.549
Positive	16.37	
Negative	17.55	

Notes: ^aA person who smoked more than 100 cigarettes in his/her past history was defined as an ever smoke. ^bOther types included large cell, sarcomatoid, and bronchoalveolar carcinoma.

(range: 2.01–92.80 µg/mL). No significant correlation between the plasma concentration of cfDNA and patient characteristics of age, sex, smoking history, histologic type, or TNM staging system was found in our study.

Discussion

It is usually difficult to obtain a tissue sample for EGFR detection because of either loss of the surgical opportunity in some advanced/metastatic patients, potential surgical complications, or economic considerations. In addition, the procedure of obtaining a tumor sample may increase the risk of cancer “seeding” to other sites.²⁷ So it is important to seek a surrogate sample for EGFR detection. It is relatively easy to obtain body fluid through minimally invasive means. In addition, it is realizable for clinical dynamic monitoring of cfDNA, which allows a potentially successful change in the treatment course, for example, acquired resistance to TKIs. Therefore, using blood for EGFR detection has been a hot spot in recent years and liquid biopsy may be megatrends of detections in the future. Most previous studies focused on advanced and metastatic patients,^{28–33} and there are few experimental data concerning EGFR mutations in the plasma of early-stage NSCLC patients.

The aim of our study was to assess EGFR mutation detection in the plasma of early-stage NSCLC patients, in an attempt to provide more convincing experimental data for clinical practice. In this study, we collected 198 plasma samples from paired patients whose tissue EGFR mutations were positive. A total of 183 (92.4%) patients were in the early stage (I_A–III_A), in whom the overall sensitivity was 17.2% versus 0%, and 36% in other two previous studies.^{19,20} We analyzed the correlation of sensitivity between the different subgroups and found that the sensitivity was positively correlated with the TNM stage, and negatively correlated with differentiation. The sensitivity of stage I_A and III_A was 1.6% and 33.3%, respectively, and that of stage IV was 50%, which is consistent with that reported in the published literature. The sensitivity of high differentiation was 0% versus 36.8% for poor differentiation, the difference being statistically different. It is therefore prudent to recommend using plasma to detect EGFR mutations in early-stage (I_A, I_B, and II_A) NSCLC patients, or those with high differentiation, because of the extremely low sensitivity. However, the sensitivity rate increased dramatically in stage III_A patients and those with poor differentiation. Therefore, blood analysis for EGFR mutations could be effectively used as a biomarker in III_A or poorly differentiated tumors for diagnosis, dynamic monitoring of cfDNA, and prediction of TKI resistance,

especially in NSCLC patients whose tissue cannot be obtained readily by surgery. However, more studies with larger sample sizes are needed to verify the reliability of plasma EGFR detection of squamous, adenosquamous, and other types (large cell, sarcomatoid, and bronchoalveolar) of lung cancer. Our ongoing research plans to assess the correlation between the plasma detection results in early-stage NSCLC and patient PFS/overall survival, hoping that it could be used to estimate the prognosis of early-stage NSCLC patients.

Tissue-based EGFR detection is the standard for prediction of clinical TKI treatment responses and prognoses. Our study found that, although 94.1% (32/34) plasma samples had identical mutations with their paired tissue samples, there were still two plasma samples whose mutation types were not detected in the paired tissues. Kimura et al^{33,34} observed the same phenomenon in their studies. The possible reason may be due to tumor heterogeneity. Gerlinger et al³⁵ demonstrated that a portion taken from different parts of a primary tumor and its metastases showed extensive inter- and intratumoral evolution. Tumor samples might not carry EGFR mutations detected in plasma. On the contrary, plasma contains comprehensive genotypic information that comes from different parts of the tumor. Traditional tissue-based EGFR detection generally ignored the complexity of the genomic mutation of the tumor. Tumoral heterogeneity highlights the difficulty of diagnosis and dictating a therapeutic course based on single tissue detection. Our study also found that 164 tissue samples tested were positive for EGFR mutations but were negative in the paired plasma samples, and most of them were from early-stage NSCLC patients. In a previous study, Ren et al²⁰ showed a similar condition. Schwarzenbach et al¹³ demonstrated that apoptotic and necrotic cells released nuclear and mitochondrial DNA into the circulation in the process of cellular destruction. It is more difficult to detect tumor-derived cfDNA because of few apoptotic and necrotic tumor cells that release EGFR DNA into blood in the early stage. So, more sensitive detection methods are needed in the future.

Our study showed no significant difference between the concentration of cfDNA and age, sex, smoking history, histologic type, or TNM stage. The exact mechanism of cfDNA remains uncertain. The mechanism can be broadly categorized as passive and active. According to the passive hypothesis, cfDNA is mainly released from healthy, inflamed, or diseased (cancerous) tissue cells undergoing apoptosis or necrosis, and rarely from apoptotic and necrotic tumor cells. For this reason, the concentration of cfDNA could not change with patient characteristics. Simultaneously, highly

sensitive measurement methods and equipment are required for detecting trace amounts of tumor-derived cfDNA and analyze the correlation between the cfDNA concentration and patient characteristics.

Plasma sample detection can provide a noninvasive way to gain genotypic information, and therefore has broad application prospects in the future. As the sample size in our study was not large enough, it was difficult to assess the plasma mutation data of adenocarcinoma and large cell carcinoma in the present study. In our ongoing prospective study, we shall use larger amounts of plasma and modify the methods for cfDNA isolation and measurement, hoping to improve the sensitivity of plasma EGFR detection. Our ongoing research plans to estimate the prognosis of early-stage NSCLC patients using plasma EGFR detection.

Conclusion

Plasma cfDNA may be a surrogate and noninvasive strategy for EGFR mutation detection in early-stage NSCLC. We recommend using plasma cfDNA as a biomarker in stage III_A or poorly differentiated tumors for gene diagnosis, especially in the patients whose tissue samples cannot be obtained by surgery. Plasma samples can really reflect the patients' EGFR mutation types. In addition, they may contain more comprehensive genotypic information that comes from different parts of the tumor than tissue specimens. Our experimental data showed plasma cfDNA concentration does not vary with the patient characteristics.

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Disclosure

The authors report no conflicts of interest in this work.

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