Non-invasive detection of EGFR and TP53 mutations through the combination of plasma, urine and sputum in advanced non-small cell lung cancer

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Abstract. The sensitivity and utility of liquid biopsy in clinical practice requires some improvement. The aim of the present study was to improve the detection of epidermal growth factor (EGFR) and cellular tumor antigen p53 (TP53) mutations in liquid biopsies from patients with advanced non-small cell lung cancer (NSCLC) by combining plasma, sputum and urine samples under the same sequencing platform. Plasma, sputum and urine samples, and tumor tissues were obtained from 50 patients with NSCLC and were analyzed using next-generation sequencing. The sensitivity of EGFR-sensitive mutation detection was 84% in plasma, 63% in sputum, 28% in urine, and 91% when combining the three liquid samples (P<0.001). The sensitivity of TP53 mutation detection increased from 87% in plasma to 94% when the three samples were combined (P<0.001). The sensitivity of EGFR or TP53 mutations detection was higher in patients with multiple metastatic sites compared with patients ≤1 metastatic site. In addition, the progression free survival (PFS) rates obtained following analysis of the three samples independently in patients with EGFR sensitizing mutations were similar, and were 9.0 months in the tissue sample, 7.5 months in plasma, 7.9 months in the sputum and 7.3 months in urine (P=0.721). The PFS of patients with TP53 mutations was shorter compared with patients without TP53 mutations and was as follows: Tissue, 8.2 months compared with 10.2 months (P=0.412); plasma, 8.4 months compared with 10.2 months (P=0.466); sputum, 8.3 months compared with 9.1 months (P=0.904); and when combined, 8.8 months compared with 10.3 months (P=0.599). The combination of plasma, sputum and urine increased the detection of EGFR or TP53 mutation with higher sensitivity, and may improve the predictive value of personalized treatment.

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Introduction

Precise molecular targeted therapy based on specific driver gene mutations has improved the prognosis of patients with advanced non-small cell lung cancer (NSCLC) (1). Mutations of driver genes, including the epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase, substitution in the serine/threonine-protein kinase B-raf V600E and alterations of the repressor of silencing 1, are used to guide targeted therapy (2). Furthermore, alterations in driver genes, including cellular tumor antigen p53 (TP53), are associated with treatment efficacy in NSCLC (3,4). Mutations of EGFR are present in 40-55 and 5-15% of lung adenocarcinoma cases in patients from Asia and Europe/USA, respectively (5,6). Examining the mutational status of EGFR, including sensitive and drug-resistant mutations such as T790M and/or TP53, may improve tumor treatment by providing targeted therapy through the use of EGFR-tyrosine kinase inhibitors (TKIs) (7,8).

Although tumor biopsy is considered as the gold standard for detecting mutations in driver genes, poor patient health status, the invasive nature of biopsies and inadequate tumor tissue samples make the completion of gene testing from tumor biopsies difficult (9,10). In particular, in patients receiving targeted therapy, it is not possible to use tissue biopsies to monitor drug resistance because of the invasive nature of the biopsy procedure. Furthermore, the underlying inter- and intratumoral heterogeneity may lead to false-negative results in gene detection (11). Liquid biopsies therefore represent an effective and non-invasive alternative to detect gene mutations, and a relatively comprehensive method to monitor changes during treatment (12,13).

It has been hypothesized that circulating tumor DNA (ctDNA) may originate from tumor cells in the peripheral

circulation and may be representative of the cancer genomic profile. The concordance rate of matched mutations in ctDNA with those in the tumor tissue DNA is >90% in certain cases (14,15). Plasma ctDNA may overcome the shortcomings of tumor heterogeneity by accurately identifying the extent of present mutations (16), and may also be used to detect drug resistance-associated mutations in driver genes prior to imaging results of computed tomography (CT) scans through dynamic monitoring during the treatment process (17). ctDNA in urine is derived from systemic circulation and may reflect the genetic information of a tumor (18-20). A previous study reported that detection of ctDNA in urine represents a non-invasive and highly accurate method of genetic profiling in patients with advanced NSCLC (21). However, some limitations exist, including the low amount of ctDNA in the circulating free DNA (~0.01% of circulating free DNA under certain circumstances, particularly for primary cancer) (22). It is therefore difficult to determine a unique genomic profile of NSCLC based on a single fluid sample. Highly sensitive assays are required to improve the genetic profiling accuracy and address this challenge (23). As ctDNA in urine and plasma have different genomic profiles and contain unique gene alterations (24), they may help to overcome the tumor heterogeneity present in a biopsy. In addition, early monitoring of dynamic changes in driver gene mutations could predict the outcome of targeted therapy and provide superior predictive value compared with imaging evaluation of CT scan (3).

Cell-free DNA (cfDNA) and tumor-associated DNA are present in the sputum of patients with NSCLC (25). Free DNA may originate from malignant cells and inflammatory cells in upper airway cancer, and may be used to detect genetic alterations (25). A previous study demonstrated that cfDNA in sputum from patients with NSCLC can be used to detect gene variations efficiently, and that the cfDNA profiles obtained from plasma, urine and sputum were different, which increased the overall rate of concurrence of mutations between cfDNA and tumor DNA significantly when the three samples were combined (26). However, to the best of our knowledge, detailed information on the consistency of individual gene detection in different sample types has not yet been described.

In the present study, the combination of plasma, sputum and urine from patients with NSCLC was used to evaluate the genomic profiles of liquid samples and tumor tissue using next-generation sequencing (NGS). Furthermore, this study aimed to evaluate the clinical performance of NGS to identify EGFR and TP53 mutations in matched pretreatment sputum, urine and plasma samples compared with tumor tissue.

Materials and methods

Patients. A total of 50 patients diagnosed with advanced NSCLC admitted at The Chinese People's Liberation Army General Hospital (Beijing, China) were recruited for the present study between October 2015 and December 2017. The inclusion and exclusion criteria for patient enrollment are stated in our previous study (26). Advanced NSCLC patients having enough tumor tissue and fluid samples including plasma, urine and sputum to analyze genetic mutations were recruited, while patients who not want to receive regular follow-up were excluded. Patients were divided into two groups as follows: i) A total of 32 patients newly diagnosed with advanced NSCLC who had not received any treatment; and ii) 18 patients with drug resistance acquired following first-line EGFR-TKI treatment and who did not receive further treatment. All patients with an EGFR sensitizing mutation detected by NGS received first-line EGFR-TKI, whereas other patients were given standard chemotherapy that consisted of cisplatin with pemetrexed for adenocarcinoma or gemcitabine with cisplatin for squamous cell carcinoma. Patients with a EGFR T790M mutation were treated with osimertinib according to guidelines (2). Signed informed consent was obtained from all patients or their families, and the present study was approved by The Ethics Committee of The Chinese People's Liberation Army General Hospital.

Sample collection and processing. Matching sputum, plasma and urine samples and tumor tissues were collected prior to first-line therapy from newly diagnosed patients or prior to changing treatment regimen from patients who had acquired drug resistance. The four types of samples were collected or extracted and stored as described previously (26). All extraction and analyses procedures were performed in a CAP/CLIA-certified diagnostic laboratory.

Library preparation and NGS. A KAPA Hyper Prep kit (Kapa Biosystems; Roche Diagnostics) was used to prepare the sequencing libraries according to the manufacturer's protocol for liquid samples (26). Targeted sequencing was conducted using GeneseeqOne™ 416-gene panel (Nanjing Geneseeq Technology Inc.). The protocol for cfDNA isolation were similar among the different liquid samples and details of the procedures were described in our previous studies (26,27).

Statistical analysis. Gene detection from tumor tissue is considered as the gold standard. The definitions of sensitivity, specificity and positive predictive value of gene alterations in each liquid sample are assessed in our previous study (26). Sensitivity is the ratio of mutations detected from liquid samples to the mutations detected from tissue sample in the same patients. The specificity is the ratio of the wild-type of driver gene detected from liquid samples to that detected from tissue samples in the same patients (28). The positive predictive value is the ratio of actual mutations to all of the mutations detected from liquid samples obtained from the same patient. The sensitivity and specificity of EGFR and TP53 mutations were compared between each liquid sample and the combination of all liquid samples was analyzed using χ^2 test. A Fisher's exact test and a one-way ANOVA with Bonferroni's post hoc test were used to compare the objective response rate (ORR) and progression free survival (PFS) for EGFR and TP53 mutation in different kinds of samples, respectively, between subgroups with or without EGFR mutations in the different types of liquid samples from patients receiving a first-line EGFR-TKI. The effect of a TP53 mutation on ORR in patients receiving a first line EGFR-TKI was analyzed using a Fisher's exact test, whereas a one-way ANOVA was used to determine the effect of TP53 mutations on PFS in patients treated with a first-line EGFR-TKI. $P<0.05$ was considered to indicate a

Table I. Clinical and demographic characteristics of patients with non-small cell lung cancer.

Characteristics	All patients, (n=50)	Newly diagnosed (n=32)	Acquired resistance (n=18)
Age, years (range)	61 (36-81)	61 (36-81)	60 (43-67)
Sex, n (%)			
Male	20 (40)	16 (50)	4 (22)
Female	30 (60)	16 (50)	14 (78)
Smoking, n (%)			
Yes	15 (30)	12 (38)	3 (17)
No	35 (70)	20 (62)	15 (83)
Histology, n (%)			
Adenocarcinoma	48 (96)	30 (94)	18 (100)
Squamous	1 (2)	1 (3)	0 (0)
Non-specific NSCLC	1 (2)	1(3)	0 (0)
Disease stage, n (%)			
IIIb	7 (14)	6 (19)	1 (6)
IV	43 (86)	26 (81)	17 (94)
Number of metastases, n (%)			
0	14 (16)	8 (25)	2 (11)
1	10 (18)	4 (12)	10 (56)
>1	26 (66)	20 (63)	6 (33)
Biopsy site for genotyping, n (%)			
Lung	47 (94)	29 (91)	10 (100)
Liver	1 (2)	1 (3)	0 (0)
Bone	1 (2)	1 (3)	0 (0)
Lymph node	1 (2)	1 (3)	0 (0)

statistically significant difference. All statistical analyses were performed using SPSS version 22 (IBM Corp.).

Results

Patient characteristics. Similarly to our previous study, 60% of participants were women, 86% had stage IV lung cancer and 96% were diagnosed with adenocarcinoma (26). Detailed information on each group of patients is presented in Table I. As previously demonstrated, the most frequently mutated genes were TP53 and EGFR, which were present in 52.2 and 48.3% of all kinds of successfully tested samples (blood, urine, sputum and tumor tissue), respectively (26). A total of 32 patients carried an EGFR sensitizing mutation and 9 patients had the T790M mutation, and 31 patients were diagnosed with TP53 mutations and 18 patients had both EGFR and TP53 mutations. The distribution of EGFR and TP53 mutations in the different types of samples are presented in Fig. 1. The appropriate therapeutic regimen was based on the genetic profiling results and each patient enrolled was followed up regularly every three months until death.

Assay characteristics of EGFR and TP53 mutations in tumor tissue and liquid biopsies. The presence of EGFR and TP53 mutations in the three liquid biopsies and in the tissue sample of patients newly diagnosed with NSCLC and of patients with acquired drug-resistant NSCLC are presented in Tables SI and SII, respectively.

The frequency of EGFR or TP53 mutations in the different types of samples is presented in Fig. 2. The mean frequency of EGFR mutations was 27.0, 8.2, 3.3 and 4.6% in tumor tissues, plasma samples, sputum samples and urine samples, respectively. Furthermore, a significant difference was observed between tumor tissue and each liquid biopsy (all $P<0.001$). In addition, a significant difference in EGFR mutations frequency was observed between tumor tissues and each type of liquid sample independently (all $P<0.001$). The mean frequency of TP53 mutations was 24.0, 7.9, 3.7 and 3.2% in tumor tissue, plasma, sputum and urine, respectively, and a significant difference in TP53 mutations frequency was observed between tumor tissues and each liquid sample independently (all $P<0.001$). The distribution of EGFR and TP53 mutations in tissue and matched liquid samples are presented in Table II. Overall, the sensitivity of detecting an EGFR sensitizing mutation was 84% for plasma, 63% for sputum and 28% for urine samples, with a combined sensitivity of 91% ($P=0.001$). In addition, the sensitivity of detecting EGFR sensitizing mutations in exons 18-20 was improved when the three types of liquid samples were combined. This sensitivity was 81% for plasma, 63% for sputum, 31% for urine and 94% when samples were combined ($P=0.003$). The sensitivity of detecting a mutation in exon 21 of EGFR gene in the different body fluids was as follows: Plasma, 86%; sputum, 67%; urine, 29%; and 90% when the three samples types were combined ($P=0.001$). Combining the three samples did not affect the sensitivity of detecting

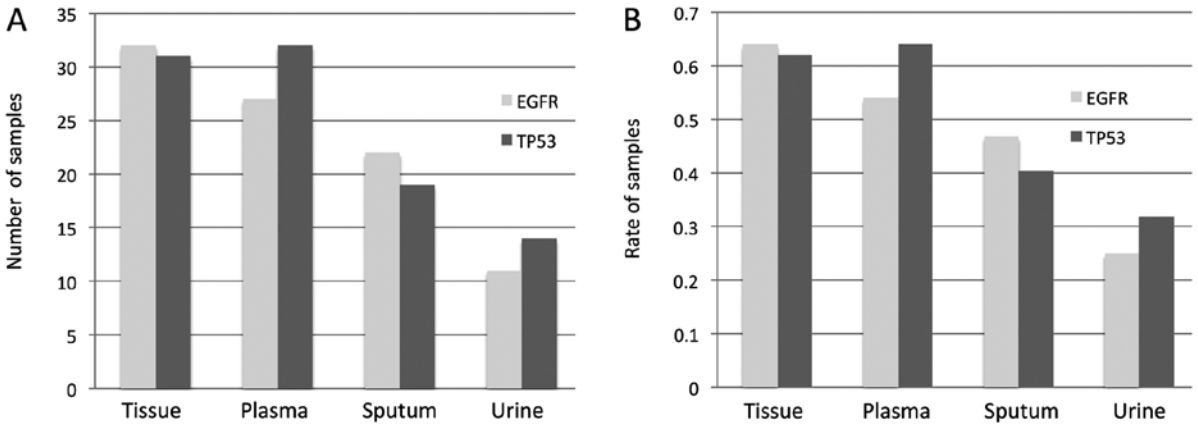

Figure 1. Distribution of EGFR and TP53 mutations in tissue, plasma, sputum and urine samples. (A) Number of EFGR or TP53 mutations, and (B) rate of EFGR or TP53 mutations in tissue, plasma, sputum and urine samples. EGFR, epidermal growth factor; TP53, cellular tumor antigen p53.

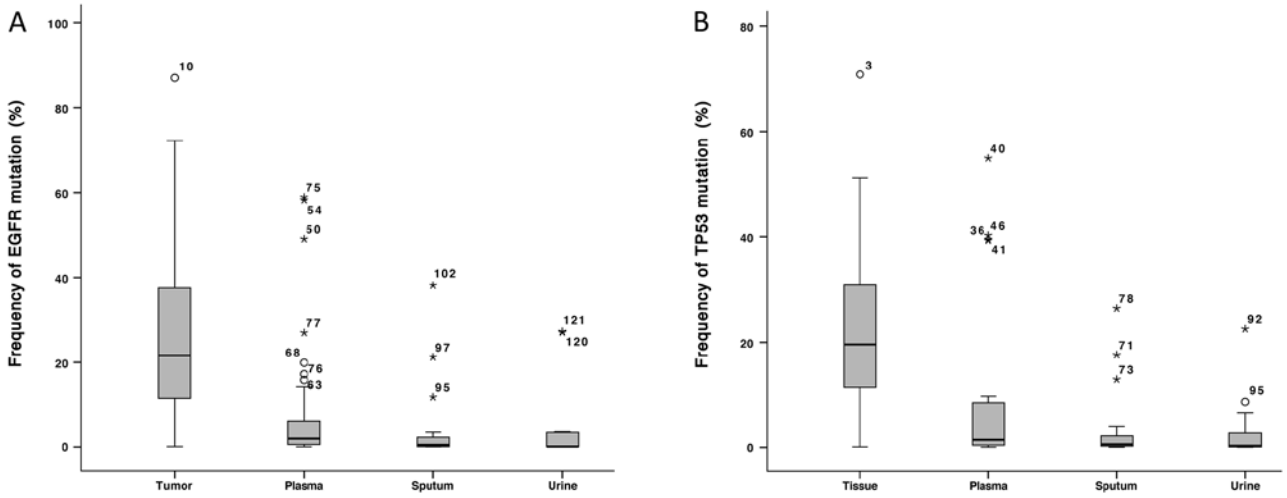

Figure 2. Frequency of EGFR and TP53 mutations in tissue, plasma, sputum and urine samples. (A) Frequency of EGFR mutations, and (B) frequency of TP53 mutations in tissue, plasma, sputum and urine samples. EGFR and TP53 mutation frequency was significantly higher in tumor samples compared with plasma, urine and sputum samples. EGFR, epidermal growth factor; TP53, cellular tumor antigen p53; ^{*,O}extreme outliers and mild outliners, respectively. Numbers on the outlier symbols represent the number of each outlier.

T790M mutation [89% (plasma) vs. 89% (combination); Table III]. Furthermore, an EGFR exon 19 deletion with a frequency of 11.8% was detected in the sputum and tumor tissue; however, it was not detected in the plasma or urine samples of newly diagnosed patient (Table SI). A patient accepted EGFR-TKIs as a first-line therapy and had a PFS of 10 months from clinical observation. In addition, T790M mutation (Table SII) was detected in plasma and sputum with frequencies of 4.84 and 0.10%, respectively; however, it was not detected in the tumor tissue of patients with drug-resistance. The patient was subsequently treated with osimertinib as second line therapy and had a PFS of 13 months.

In all patients enrolled in the present study, the sensitivity of TP53 mutation detection was 87% in plasma samples, 45% in sputum samples and 26% in urine samples. By combining the three body fluid samples, the sensitivity of TP53 mutation detection increased to 94% (P=0.001). In the newly diagnosed patients, the sensitivity of TP53 mutations in plasma, urine and sputum sample was 90, 48 and 33%, respectively, and the combined sensitivity increased to 95% (P=0.001). In the drug-resistant group, the sensitivity also significantly increased from 80% in the plasma sample to 90% when all samples were combined (P=0.002; Table III).

Predictors of EGFR and TP53 mutations detection sensitivity. To further examine the factors affecting the detection sensitivity of driver gene mutations in the different body fluid samples, a subgroup analysis based on the clinical characteristics of patients was performed. The results demonstrated an association between the number of metastases and the detection sensitivity of EGFR and TP53 mutations (Table IV). The sensitivity of detecting EGFR sensitizing mutations in the group with >1 metastases was greater compared with that in the group with ≤1 metastasis and was as follows: Sputum, 77% compared with 36% (P=0.025); urine, 42% compared with 0% (P=0.009); and combined, 100% compared with 73% (P=0.021). Detection sensitivity for TP53 mutations in urine was also higher in patients with >1 metastases compared with that in patients with ≤1 metastasis (39% compared

Table II. Number of EGFR and TP53 mutations in tissue and matched liquid samples.

	Plasma			Sputum			Urine			Combination		
Mutation	+	-	Total	+	-	Total	+	-	Total	+	-	Total
EGFR E18-20 sensitizing mutation, n												
+	13	3	16	10	6	16	5	11	16	15	1	16
-	0	34	34	0	31	31	0	28	28	0	34	34
Total	13	37	50	10	37	47	5	39	44	15	35	50
EGFR E21 sensitizing mutation, n												
+	18	3	21	14	7	21	6	15	21	19	2	21
-	1	28	29	1	25	26	0	23	23	1	28	29
Total	19	31	50	15	32	47	6	38	44	20	30	50
EGFR T790M, n												
+	8	1	9	6	3	9	3	6	9	8	1	9
-	1	8	9	1	8	9	0	9	9	1	8	9
Total	9	9	18	7	11	18	3	15	18	9	9	18
TP53, n												
+	27	4	31	14	17	31	7	20	27	29	2	31
-	5	14	19	4	12	16	4	13	17	9	10	19
Total	32	18	50	18	29	47	11	33	44	38	12	50

EGFR, epidermal growth factor; TP53, cellular tumor antigen p53; +, represents positive mutations; -, represents negative mutations.

with 0%; P=0.041). However, there was no significant difference in sensitivities based on sex, age and smoking history.

Prognostic predictions of first-line EGFR-TKIs efficiency based on cfDNA. In the newly diagnosed group, 15 patients were identified as having tumors with EGFR sensitizing mutations and therefore received a first-line EGFR-TKI treatment. Among these patients, the ORRs were 67, 67, 63 and 66% in the tissue, plasma, sputum and urine, respectively, and 75% when the three types of samples were combined (P=0.981). The PFS times were similar in patients with EGFR sensitizing mutations in tissue (9.0 months), plasma (7.5 months), sputum (7.9 months) and urine (7.3 months; P=0.721; Table V). The ORR values were 56% in the tissue samples from patients with TP53 mutations and 83% in the tissue samples from patients without TP53 mutations (P=0.580). The ORR values were 60% in the plasma samples from patients with TP53 mutations and 80% in the plasma samples from patients without TP53 mutations (P=0.6). The ORR values were 64% in the combined liquid samples from patients with TP53 mutations and 75% in the combined liquid samples from patients without TP53 mutations (P=0.566). By excluding the group of patients with TP53 mutations in the urine samples, as patients with or without TP53 mutation in urine had the same PFS, the PFS times of patients with TP53 mutations were decreased compared with the patients without TP53 mutations, and were as follows: Tissue, 8.2 months compared with 10.2 months (P=0.412); plasma, 8.4 months compared with 10.2 months (P=0.466); sputum, 8.3 months compared with 9.1 months (P=0.904); and when combined, 8.8 months compared with 10.3 months (P=0.599; Table VI). However, there was no significant difference in these comparisons. The DCR of patients receiving first-line EGFR-TKI among different groups also demonstrated no significant difference (Tables V and VI).

Discussion

In the present prospective study, the combination of plasma, sputum and urine biopsies with matching tumor tissues from patients with NSCLC was used for the first time to detect mutations in EGFR and TP53 using a NGS platform. The present study determined the association of EGFR and TP53 mutations in plasma, sputum, urine and tumor tissue and demonstrated the value of combining samples for gene detection, with the prediction efficacy of first line EGFR-TKI therapy predicted by EGFR and TP53 gene variations detected through the different types of samples. The results demonstrated that the differences in the mean frequency of EGFR or TP53 mutation in the plasma, sputum and urine were not statistically significant compared with the tumor tissue, which suggested that liquid samples may potentially be used to detect gene variations. Furthermore, the present study reported that the combination of these three body fluid samples increased the sensitivity of detecting EGFR sensitizing mutations and TP53 mutations. The sensitivity of combining liquid biopsy was improved compared with previous studies that used single liquid sample (29-31). The present study provided an effective method to detect mutations in common driver genes, including EGFR and TP53, with a relatively high sensitivity and specificity in patients with advanced NSCLC.

In the present study, the sensitivity of detecting EGFR sensitizing mutations in sputum and urine cfDNA was associated with the number of metastatic sites. Previous studies

Table III. Sensitivity, specificity and positive predictive value of EGFR and TP53 mutations in liquid samples compared with tissue samples.

A, Sensitive mutation in exons 18/19/20 of EGFR

	Sensitivity, % (95% CI)					Specificity, % (95% CI)					Positive predictive value, % (95% CI)				
Treatment stage	Plasma	Sputum	Urine	All	P-value	Plasma	Sputum	Urine	All	P-value	Plasma	Sputum	Urine	All	P-value
Newly diagnosed	60 (17-93)	40 (7-83)	0 (0-54)	80 (30-99)	0.070	100 (85-100)	100 (85-100)	100 (85-100)	100 (85-100)	/	100 (31-100)	100 (20-100)	/	100 (40-100)	/
Drug-resistant	91 (57-100)	73 (39-93)	45 (18-75)	100 (68-100)	0.018	100 (60-100)	100 (60-100)	100 (60-100)	100 (60-100)	/	100 (66-100)	100 (60-100)	100 (46-100)	100 (68-100)	/
All	81 (54-95)	63 (36-84)	31 (12-59)	94 (68-100)	0.003	100 (88-100)	100 (88-100)	100 (88-100)	100 (88-100)	/	100 (72-100)	100 (66-100)	100 (46-100)	100 (73-100)	/

B, Sensitive mutation in 21 exon of EGFR

	Sensitivity, % (95% CI)					Specificity, % (95% CI)					Positive predictive value, % (95% CI)				
Treatment stage	Plasma	Sputum	Urine	All	P-value	Plasma	Sputum	Urine	All	P-value	Plasma	Sputum	Urine	All	P-value
Newly diagnosed	93 (64-100)	57 (30-81)	36 (14-64)	93 (64-100)	0.001	100 (81-100)	100 (81-100)	100 (81-100)	100 (80-100)	/	100 (72-100)	100 (60-100)	100 (46-100)	100 (72-100)	/
Drug-resistant	71 (30-95)	86 (42-96)	14 (1-60)	86 (42-96)	0.012	91 (57-100)	91 (57-100)	100 (68-100)	91 (57-100)	0.615	83 (36-99)	86 (42-96)	100 (5-100)	86 (42-96)	0.008
All	86 (63-96)	67 (43-85)	29 (12-52)	90 (68-98)	0.001	97 (82-100)	97 (82-100)	100 (87-100)	97 (82-100)	0.626	95 (72-100)	93 (66-100)	100 (52-100)	95 (73-100)	0.874

C, T790M of EGFR

	Sensitivity, % (95% CI)					Specificity, % (95% CI)					Positive predictive value, % (95% CI)				
Treatment stage	Plasma	Sputum	Urine	All	P-value	Plasma	Sputum	Urine	All	P-value	Plasma	Sputum	Urine	All	P-value
Drug-resistant	89 (51-99)	56 (23-85)	33 (9-69)	89 (51-99)	0.024	89 (51-99)	89 (51-99)	100 (63-100)	89 (51-99)	0.612	89 (51-99)	83 (36-99)	100 (31-100)	89 (51-99)	0.832

D, TP53 mutation

	Sensitivity, % (95% CI)					Specificity, % (95% CI)					Positive predictive value, % (95% CI)				
Treatment stage	Plasma	Sputum	Urine	All	P-value	Plasma	Sputum	Urine	All	P-value	Plasma	Sputum	Urine	All	P-value
Newly diagnosed	90 (68-98)	48 (26-70)	33 (15-57)	95 (74-100)	0.001	73 (39-93)	64 (32-88)	64 (32-88)	36 (12-68)	0.336	86 (64-96)	72 (42-90)	64 (32-88)	74 (53-88)	0.474
Drug-resistant	80 (44-96)	40 (14-73)	20 (4-56)	90 (54-99)	0.002	75 (36-96)	83 (36-99)	88 (47-99)	75 (36-96)	0.841	80 (44-96)	86 (42-99)	75 (22-99)	82 (48-97)	0.959
All	87 (69-96)	45 (28-64)	26 (12-47)	94 (77-100)	0.001	74 (49-90)	75 (47-92)	76 (50-92)	53 (29-75)	0.404	84 (66-94)	74 (49-90)	64 (36-86)	76 (59-88)	0.504

CI, confidence interval; EGFR, epidermal growth factor; TP53, cellular tumor antigen p53; /, value could not be analyzed.

Table IV. Association between sensitivity of detection for EGFR and TP53 mutations and clinical characteristics.

A, Sensitizing mutation in EGFR

Characteristic	Plasma	Sputum	Urine	Total
Sex				
Male, n (%)	8 (89)	6 (67)	5 (56)	9 (100)
Female, n (%)	23 (82)	18 (64)	6 (21)	25 (89)
P-value	0.523	0.614	0.066	0.422
Number of metastases, n				
>1, n (%)	23 (88)	20 (77)	11 (42)	26 (100)
≤1, n (%)	8 (73)	4 (36)	0 (0)	8 (73)
P-value	0.236	0.025	0.009	0.021
Smoking				
Yes, n (%)	7 (88)	6 (75)	4 (50)	8 (100)
No, n (%)	24 (83)	18 (62)	7 (24)	26 (90)
P-value	0.560	0.409	0.163	0.470
Age, years				
>60, n (%)	6 (86)	6 (86)	1 (14)	6 (86)
≤60, n (%)	25 (83)	18 (60)	10 (33)	28 (93)
P-value	0.685	0.204	0.310	0.477

B, TP53 mutation

Characteristic	Plasma	Sputum	Urine	Total
Sex				
Male, n (%)	15 (94)	7 (44)	5 (31)	16 (100)
Female, n (%)	12 (80)	7 (47)	4 (27)	13 (87)
P-value	0.275	0.578	0.546	0.226
Number of metastases, n				
>1, n (%)	21 (91)	12 (52)	9 (39)	22 (96)
≤1, n (%)	6 (75)	2 (25)	0 (0)	7 (88)
P-value	0.268	0.180	0.041	0.456
Smoking				
Yes, n (%)	14 (93)	7 (47)	5 (33)	15 (100)
No, n (%)	13 (81)	7 (44)	4 (25)	14 (88)
P-value	0.325	0.578	0.454	0.258
Age, years				
>60, n (%)	8 (80)	4 (40)	4 (40)	9 (94)
≤60, n (%)	19 (90)	10 (48)	5 (24)	20 (95)
P-value	0.387	0.497	0.302	0.548

EGFR, epidermal growth factor; TP53, cellular tumor antigen p53.

demonstrated that an increased number of metastatic sites is associated with increased cfDNA present in the circulation (32,33). Increased cfDNA in circulation from tumor cells may therefore be associated with the upper limit of the assay sensitivity. The present study demonstrated that cfDNA was also present in urine and sputum and that the detection sensitivity of EGFR or TP53 mutations in sputum or urine was higher in patients with >1 metastatic site.

The ORR following first-line EGFR-TKIs was similar in patients with EGFR sensitizing mutations detected in plasma, sputum, urine and tissue samples. There was no significant difference in the PFS in patients receiving first-line EGFR-TKIs among patients with mutations of EGFR, EGFR mutations in the plasma samples, EGFR mutations in the sputum and EGFR mutations in the urine. These results suggested that the value of EGFR mutations detected in liquid samples was similar to the value detected in tissue samples and that they may both be used to predict the efficacy of first line EGFR-TKIs. Previous studies demonstrated that the ORR was 65.7-75.0% for first-line EGFR-TKIs in patients with an EGFR mutation detected in plasma or serum cfDNA (34,35). In the present study, results from urine and sputum samples indicated that additional liquid samples may be useful in clinical practice for genetic profiling, and may help to determine which therapy would be best, in particular in patients for whom it may not be possible to obtain a tissue biopsy. The ORR for first line EGFR-TKI in patients with TP53 mutations was decreased compared with patients without TP53 mutations in the tumor biopsy, plasma samples and when the liquid samples were combined. When evaluating the PFS of first line EGFR-TKIs, excluding urine samples, TP53 mutations in tumor and liquid biopsies predicted a decreased PFS. TP53 mutations were associated with lower disease control rate and ORR in patients receiving first-line EGFR-TKIs based on the tissue or plasma samples. The results from the present study were similar to previous studies that demonstrated that TP53 mutations detected in tumor tissues or plasma samples were associated with poor ORR and shorter PFS, and that TP53 mutations detection in sputum was possible and could be used to predict the efficacy of first-line EGFR-TKIs (3,4,8). However, the aforementioned differences were not statistically significant in the present study, which may be due to the small sample size.

To the best of our knowledge, cfDNA from sputum was used for the first time to detect gene variations in the present study. The detection sensitivity of EFGR sensitizing mutations using sputum was lower compared with that obtained when using plasma samples; however, it was higher than when using urine samples. The ORR of treatment with EGFR-TKI in patients with EGFR mutations detected in the sputum was similar to that of patients with EGFR mutations detected in the tissue samples. Furthermore, certain EGFR sensitizing mutations, including an EGFR exon 19 deletion, were not detected in the plasma or urine samples; however, they were detected in the cfDNA from sputum in a newly diagnosed patient. These results demonstrated that combining cfDNA from different body fluid samples may improve the sensitivity of liquid biopsy, enabling the detection of more sensitive mutations using fluid samples and allowing more patients to receive individualized treatment.

Similar to previous studies, driver gene mutations, including EGFR and TP53, were not detected in tumor tissues, but were detected in body fluid samples (36,37). Previous studies reported inconsistent results between tissue biopsies and liquid biopsies, where certain driver gene mutations were identified in liquid samples but not in tumor tissue; however, this may be due to tumor heterogeneity and clonal hematopoiesis (CH) (38,39). In the present study, as the genomic DNA from peripheral blood leukocytes was also included in the

Table V. Prediction of first-line EGFR-tyrosine kinase inhibitor therapy effect based on EGFR mutation status in patients newly diagnosed with non-small cell lung cancer.

Variable	Tissue	Plasma	Sputum	Urine	Combination	P-value
EGFR mutation, n (%)	15 (47)	12 (38)	8 (30)	3 (13)	13 (41)	0.044
ORR, n (%)	10 (67)	8 (67)	5 (63)	2 (66)	9 (75)	0.981
DCR, n (%)	13 (87)	11 (91)	7 (88)	3 (100)	12 (92)	0.903
PFS, months	9.0	7.5	7.9	7.3	9.3	0.721

EGFR, epidermal growth factor; ORR, objective response rate; DCR, disease control rate; PFS, progression free survival.

Table VI. Prediction of first-line EGFR-tyrosine kinase inhibitor therapy effect based on TP53 mutation status in patients newly diagnosed with non-small cell lung cancer.

Sample type	ORR, n (%)	DCR, n (%)	PFS, months
Tissue			
Wild-type	5 (83)	6 (100)	10.2
Mutant	5 (56)	7 (78)	8.2
P-value	0.580	0.586	0.412
Plasma			
Wild-type	4 (80)	5 (100)	10.2
Mutant	6 (60)	8 (80)	8.4
P-value	0.600	0.524	0.466
Sputum			
Wild-type	6 (60)	8 (80)	9.1
Mutant	4 (80)	5 (100)	8.8
P-value	0.600	0.524	0.904
Urine			
Wild-type	6 (60)	8 (80)	8.3
Mutant	4 (80)	5 (100)	10.4
P-value	0.600	0.524	0.393
Combination[a]			
Wild-type	3 (75)	4 (100)	10.3
Mutant	7 (64)	9 (82)	8.8
P-value	1.000	1.000	0.599

[a]Combination of plasma, sputum and urine samples. EGFR, epidermal growth factor; TP53, cellular tumor antigen p53; ORR, objective response rate; DCR, disease control rate; PFS, progression free survival.

sequencing analysis as a background reference, but excluded when the result was analyzed, the probability of inconsistent results caused by CH was reduced. As most patients enrolled in the present study had >1 metastasis site, a single biopsy of the tumor tissue did not reflect the overall tumor genomic profile. Therefore, cfDNA from body fluids came from multiple disease sites throughout the body, which may allow the detection of a more diverse and representative genomic profile than from single tumor biopsy (36). In the present study, tumor heterogeneity may have been the primary cause of the inconsistent results observed in gene detection from body fluid samples and tumor tissues. For example, one case of T790M mutation was detected in the plasma and sputum, but was not detected in the tumor tissue; the patient received osimertinib treatment and had a PFS of 13 months, which confirmed the clinical efficacy of the liquid biopsy. Subsequently, the results from the liquid biopsy that were inconsistent with the tissue biopsy may not always be a false positive. Instead, the tissue biopsy may be a false negative due to tumor heterogeneity.

In conclusion, the present study confirmed that the use of target sequencing with HiSeq4000 and the GeneSeqOne™ gene panel may be used to detect with a high sensitivity EGFR and TP53 mutations in cfDNA from plasma, sputum and urine samples of patients with advanced NSCLC. Although the sensitivity based on liquid samples individually may not meet the needs of clinical practice, it was possible to detect that the genomic profiles were distinct from each other, and that the combination of results from liquid samples increased the overall sensitivity of EGFR and TP53 mutations. In addition, liquid samples provided a method to predict the efficacy of a personalized therapeutic regimen. Further investigation will require a bigger sample size in order to validate the differences between genomic profiles in unique liquid samples and the utility of combining liquid biopsy.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

ZW and ZY contributed equally to the conception and design of the present study. ZW and ZY analyzed and interpreted data and wrote the manuscript. YD, QZ, DHC and KLM selected patients, collected liquid and tissue samples and completed the follow-up of the recruited patients. CSL, WZ and ZXL performed the experimental procedures. JZ contributed to statistical analysis. LAC conceived and designed the study and revised the manuscript critically for intellectual content. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethical Committee of the Chinese People's Liberation Army General Hospital (Beijing, China; approval no. S2015-099-001). All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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