Clin. Lab. 2019;65:XXX-XXX

ORIGINAL ARTICLE

Evaluation of PAP in *EGFR* Mutational Testing in Advanced NSCLC: a Comparative Study

Chen Jinliang [1, *], Xu Yang [2, *], Xu Xiaoyan [3, *], Su Yudong [1], Wang Xinyue [1], Meng Zhaoting [1], Lin Li [1], Sun Ning [1], Guo Hua [1], Huang Dingzhi [1], Zhang Lun [1], Chen Peng [1]

** These authors contributed equally to this work*
[1] Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center for Cancer, Key Laboratory of Cancer Prevention and Therapy, Tianjin, China
[2] Department of the Graduate School, Tianjin Medical University, Tianjin, China
[3] Department of Oncology, Inner Mongolia People's Hospital, Hohhot, Inner Mongolia Autonomous Region, China

SUMMARY

Background: ***EGFR*** **mutational testing is crucial for advanced non-squamous NSCLC. PAP is a sensitive and selective method to detect rare mutations.**
Methods: **Eighty-five patients with non-squamous NSCLC were enrolled in this study. A set of paired plasma samples from each patient were collected and detected by PAP and ARMS.**
Results: **Of 85 paired samples, 78.8% (67/85) presented the same mutational status by the two methods. There was no statistically significant difference between the mutation frequencies in plasma samples detected with PAP and ARMS (p = 0.096).**
Conclusions: **PAP technology appears to be an alternative choice with relatively high sensitivity for the detection of plasma** ***EGFR*** **mutations.**
(Clin. Lab. 2019;65:xx-xx. DOI: 10.7754/Clin.Lab.2019.190216)

Correspondence:
Prof. Zhang Lun
Department of Maxillofacial and
Otorhinolaryngological Oncology
Tianjin Medical University Cancer Institute and Hospital
National Clinical Research Center for Cancer
Key Laboratory of Cancer Prevention and Therapy
Tianjin 300060
China
Email: zhanglun@tjmuch.com

Prof. Chen Peng
Department of Thoracic Oncology
Tianjin Medical University Cancer Institute and Hospital
National Clinical Research Center for Cancer
Key Laboratory of Cancer Prevention and Therapy
Tianjin 300060
China
Email: chenpengdoc@sina.com

KEY WORDS

circulating free DNA, EGFR, pyrophosphorolysis-activated polymerization, amplification refractory mutation system

LIST OF ABBREVIATIONS

ARMS - amplification refractory mutation system
cfDNA - circulating free DNA
ddPCR - droplet digital PCR
EGFR - epidermal growth factor receptor
EGFR-TKI - EGFR-tyrosine kinase inhibitor
NGS - next-generation sequencing
NSCLC - non-small-cell lung cancer
PAP - pyrophosphorolysis-activated polymerization

Manuscript accepted June 29, 2019

INTRODUCTION

Non-small-cell lung cancer (NSCLC) accounts for about 85% of lung cancer [1] and remains the main cause of mortality among patients with cancer worldwide [2]. Currently patients with NSCLC, especially for the non-squamous subtype [3], are strongly recommended to have genetic analysis, which is associated with the efficacy of targeted therapy, according to clinical guidelines. Epidermal growth factor receptor (*EGFR*) mutations are the first discovered mutations in NSCLC and show relatively high frequencies in Asian patients [4,5]. Therefore, it is of great importance to detect the *EGFR* mutations with available methods from samples including tissue biopsy, peripheral blood, pleural effusion, and so on. Conventionally, tissue specimens were utilized as the primary source for mutational testing. However, the majority of NSCLC patients did not have enough tissue samples or cytological samples, even in well-designed studies [6,7]. Faced with these limitations, liquid biopsy emerged as a surrogate source and represented a fundamental tool. Circulating free DNA (cfDNA) in the blood was discovered 7 decades ago [8]. In recent years, the values of cfDNA have been widely explored as a noninvasive means for predicting response and resistance to EGFR inhibitors [9-11]. Nowadays, plasma cfDNA has been approved for genetic analysis with high specificity [12].

A variety of methods can be used for mutational testing in cfDNA, e.g., high-resolution melt (HRM) analysis, next-generation sequencing (NGS), multiplexed real-time reverse transcription (RT)-PCR based assays [13]. Nowadays the following methods are commonly utilized: ARMS, droplet digital PCR (ddPCR) and NGS. With limitations in clinical practice, there is no standardized method for cfDNA isolation and analysis. Here, we examined the value of a method, namely PAP, in *EGFR* mutation testing. PAP is a sensitive and selective method for DNA amplification to detect rare mutations. In this method, DNA polymerization and its reverse reaction, pyrophosphorolysis, are serially coupled by polymerase. By pyrophosphorolysis, activable oligonucleotides which blocked at 3' terminal are removed, allowing extension to occur. In theory, PAP has a potential analytical selectivity of 3.3×10^{11} [14] (Figure 1). In this study, PAP was assessed by detecting *EGFR* mutations including 19Del and L858R, which were sensitive mutations and accounted for 85% ~ 90% *EGFR* mutations. The T790M mutations were also involved because its meaning and frequency in acquired resistance to EGFR-tyrosine kinase inhibitors (EGFR-TKIs) therapy [15]. To evaluate the clinical performance of PAP, the conventional ARMS served as a standard method for comparison.

MATERIALS AND METHODS

Study population

This was a prospective study approved by the Ethics Committee of the Cancer Institute and Hospital of Tianjin Medical University (Approval number: E2018086). Eighty-five patients with non-squamous NSCLC were enrolled between March 2016 and June 2017. All participants provided informed consent prior to study entry. Peripheral blood from these patients was collected and relevant information was obtained including age, gender, smoking history, and disease staging. Patients were excluded from the study if pregnant and those who were aged under 18 or over 75 years were also excluded from this study. TNM staging was done according to the 8th Edition of the TNM Classification [16]. The characteristics of 85 patients in this study are shown in Table 1.

Sample collection

A set of paired blood samples from each patient were collected into 2 tubes with EDTA-K2 anticoagulant (10 mL purple-capped). All samples were processed at room temperature within 2 hours from the time of blood extraction. Plasma was separated from the cellular fraction by centrifugation (Eppendorf centrifuge 5810R) at 1,500 g/4°C for 10 minutes. The supernatant was collected and transferred into 1.5 mL Eppendorf tubes. Then plasma was frozen at -80°C until cfDNA extraction. For extraction, samples were thawed at 4°C and then were centrifuged at 5,000 g for 20 minutes to ensure that impurities of the supernatant were removed. cfDNA of all samples was isolated with Human Plasma Circulating Nucleic Acid Extraction Kit (GENETIC BIOTEK Co., Ltd, Tianjin, China).

EGFR mutation assessment

Here, *EGFR* mutations in cfDNA including 19Del, L858R, T790M, and wild-type were detected by PAP technology with *EGFR* Mutations Detection kit (based on real-time PAP nucleic acid amplification) (GENETIC BIOTEK Co., Ltd, Tianjin, China). In addition, plasma *EGFR* mutations were detected by ARMS, which served as a routine method, with the Human *EGFR* Gene Mutations Fluorescence PCR Diagnostic Kit (ADx-ARMS®, Amoy Diagnostics Co., Ltd, Xiamen, China), which had passed ISO13485 QMS and obtained CE certification and CFDA registration.

Mutation-specific primers were used for target amplification. The mutant amplicons were detected with a novel fluorescent probe with which even 1% mutant DNA in the background of wild type genomic DNA could be detected.

PAP was conducted with the conditions as follow: preincubation at 96°C for 2 minutes, first-round amplification (96°C for 12 seconds, 64°C for 30 seconds, followed by 68°C for 60 seconds) for 40 cycles, followed by a temperature increasing program from 68°C to 95°C by 0.5°C every 5 seconds. As for ARMS, the following

Table 1. Characteristics of 85 patients.

Characteristic	Value
Age (years), median (range)	62 (32 - 80)
Gender, n (%)	
Male	47 (55.3)
Female	38 (44.7)
Smoking status, n (%)	
Never	51 (60.0)
Former	34 (40.0)
Family history, n (%)	
Yes	25 (29.4)
No	60 (70.6)
ECOG, n (%)	
0	2 (2.4)
1	45 (52.9)
2	38 (44.7)
Histology, n (%)	
ADC	78 (91.8)
Non-ADC	7 (8.2)
Stage, n (%)	
IIIA	4 (4.7)
IIIB	4 (4.7)
IVA	19 (22.4)
IVB	58 (68.2)

*** ADC - adenocarcinoma, Non-ADC - adenosquamous carcinoma, large cell carcinoma, sarcomatoid carcinoma.**

conditions were based on its protocol: pre-incubation at 95°C for 5 minutes, first-round amplification (95°C for 25 seconds, 64°C for 20 seconds, followed by 72°C for 20 seconds) for 15 cycles, followed by second round amplification (93°C for 25 seconds, 60°C for 35 seconds, followed by 72°C for 20 seconds) for 31cycles.

Data analysis and statistics

McNemar's test was used to analyze the concordance of mutant abundance detected by PAP and ARMS. Chi-square test was used to analyze the correlations between *EGFR* status and characteristics. All statistical tests were two sided and results were considered significantly different if $p < 0.05$. Statistical analysis was performed using SPSS Version 24.0 (IBM Corp., Armonk, NY, USA).

RESULTS

A total of 170 plasma samples from 85 patients were analyzed. 55% patients were male, 60% had never smoked, 90.6% had stage IV disease, and 91.8% were classified as adenocarcinomas. 49.4% could provide tissue samples. 25.9% underwent EGFR-TKI treatment.

Comparison of the clinical performance of PAP with ARMS

Both PAP and ARMS were performed to detect the plasma *EGFR* mutations in this study. Of 85 paired samples, 78.8% (67/85) presented the same mutational status by the two methods. The sensitivity and specificity for plasma *EGFR* mutation detection by PAP were 53.5% (15/28) and 91.2% (52/57), respectively. There was no statistically significant difference between the mutation frequencies of samples detected with PAP and ARMS ($p = 0.096$) using McNemar's test (Table 2). For 19Del, the sensitivity, specificity, the positive predictive value, and the negative predictive value were 50.0% (8/16), 98.5% (67/68), 80.0% (8/10), and 89.3% (67/75), respectively. For L858R, the sensitivity, specificity, the positive predictive value, and the negative predictive value were 66.7% (6/9), 93.4% (71/76), 54.5% (6/11), and 96.0% (71/74), respectively. For T790M, the sensitivity, specificity, the positive predictive value, and the negative predictive value were 57.1% (4/7), 100% (78/78), 100% (4/4), and 96.3% (78/81), respectively (Table 3).

Comparison of inconsistent plasma EGFR status with tissue status

The paired testing results of 9 patients were included in the analysis of this part. Inconsistency between the two methods did exist. Although in plasma samples 21.2% (18/85) of patients showed different *EGFR* status by the two methods, only half of them had a match on the tissue samples after excluding the effects of EGFR-TKIs. Of note, here *EGFR* status in tumor tissue was detected by Sanger sequencing. All these available results were listed in Table 4. With tumor tissue *EGFR* as the gold standard, the concordance rate of ARMS was 66.7% while that of PAP was 22.2%.

Correlations between plasma EGFR mutation load and characteristics

Since PAP appeared to be a reliable method, further analysis was performed to detect whether there was a positive correlation between the *EGFR* mutation load and clinical pathological characteristics. As shown in Table 5, a positive correlation between mutation load and gender was observed ($p = 0.037$), indicating that the rate of detection of *EGFR* mutations was significantly higher in female patients than in male cases. However, no positive correlation between *EGFR* mutation load and the other characteristics was found, including smoking status, family history, ECOG and so on.

Table 2. Concordance of plasma *EGFR* mutation status between PAP and ARMS.

PAP	ARMS		Total	p
	Mutant	Wild		
Mutant	15	5	20	
Wild	13	52	65	
Total	28	57	85	0.096

Table 3. *EGFR* mutations detected with PAP and ARMS.

PAP	ARMS						Total
	19Del	L858R	T790M	19Del + T790M	L858R + T790M	Wild Type	
19Del	5	0	0	0	0	2	7
L858R	0	5	1	0	0	4	10
19Del + T790M	0	0	0	3	0	0	3
L858R + T790M	0	0	0	0	1	0	1
Wild Type	7	2	0	1	1	53	64
Total	12	7	1	4	2	59	85

Table 4. Inconsistent plasma *EGFR* status of 9 patients with paired tissue samples.

Case	*EGFR* Status		
	Plasma (PAP)	Plasma (ARMS)	Tissue (Sanger)
1	Wild-type	19Del	19Del
2	Wild-type	19Del	19Del
3	Wild-type	19Del	19Del
4	Wild-type	19Del	19Del
5	Wild-type	L858R T790M	L858R
6	L858R	Wild-type	Wild-type
7	19Del	Wild-type	Wild-type
8	19Del	Wild-type	19Del
9	Wild-type	L858R	Wild-type

DISCUSSION

In this single-center study in a real-life setting, we examine the value of PAP for *EGFR* mutational testing in advanced non-squamous NSCLC. This became the novel aspect of this study. In addition, we listed the inconsistent plasma *EGFR* status and made a comparison with corresponding tissue status.

To design this study, we mainly focused on the assessment of the method PAP in *EGFR* mutational testing. As this study was performed in a single center, some inherent limitations were difficult to avoid. In this study, only 49.4% of patients provided tissue samples, thus tissue status was not sufficient for genetic analysis. Since liquid biopsy had already served as common supplement, here plasma samples were mainly collected. Beyond positive mutations, examination of the specificity of PAP, *EGFR* wild-type was also included in the cf-DNA testing. Moreover, in order to get enough patients enrolled, patients were not well distinguished based on EGFR-TKI treatment. However, this factor would be taken into account when the tissue status was involved in the analysis, because it was well established that *EGFR* resistance mutations might be acquired due to

Table 5. Correlations between plasma EGFR mutation load and characteristics.

Characteristics	PAP		χ^2	p
	Mutant	Wild-type		
Gender				
Male	7	40	4.357	0.037
Female	13	25		
Smoking Status				
Never	5	29	2.452	0.117
Former	15	36		
Family History				
Yes	8	17	1.412	0.235
No	12	48		
ECOG				
0	1	1	-	0.328
1	12	33		
2	7	31		
Histology				
ADC	20	58	-	0.191
Non-ADC	0	7		
Stage				
IIIA	2	2	-	0.483
IIIB	0	4		
IVA	4	15		
IVB	14	44		

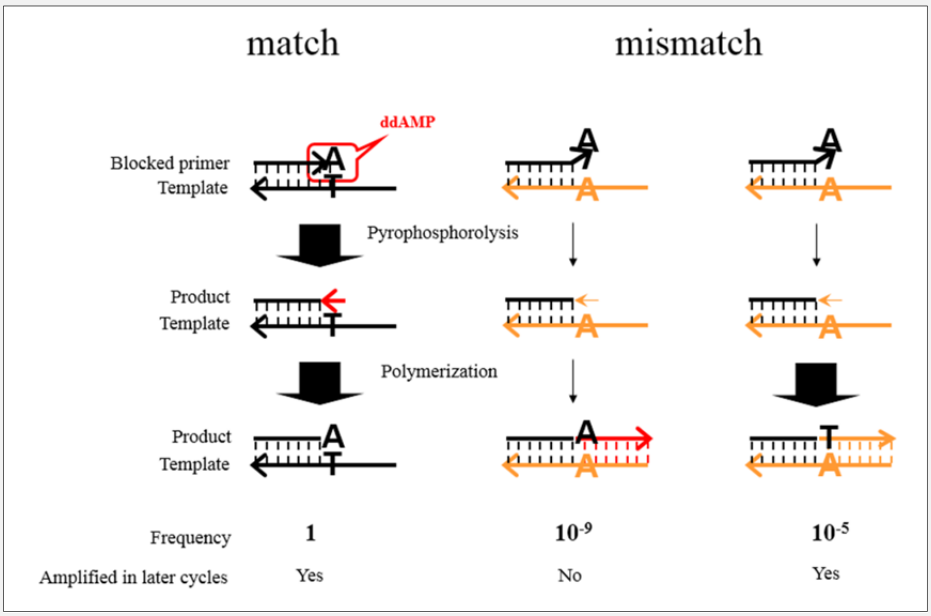

Figure 1. Schematic representation of the principle of PAP.

EGFR-TKI treatment [17].

As a classic method of mutational testing, PAP differs from PCR in following ways: necessary substrates, working principle, mismatch probability, etc. [14]. However, little is known about the difference between PAP and ARMS in clinical performance. In a previous study, PAP technology had been developed and validated in testing ultrarare somatic mutations in the human TP53 gene [18]. Additionally, PAP assay was also utilized in detecting microinsertions/deletions/indels in non-metastatic breast cancer through monitoring *EGFR* mutations [19]. PAP was initially developed to enhance the specificity of allele-specific PCR for detection of known mutations in the presence of a great excess of wild-type allele. The specific oligonucleotides have the novel and unexpected property of high specificity to mismatches with the template throughout lengths of the oligonucleotides. Thus, PAP also can form the basis of microarray-based scanning or resequencing methods to detect virtually all mutations. Comparing with ARMS, we examined the value of PAP in *EGFR* mutational testing. In this study, we found that a total of 67 (78.8%) patients presented the same mutational status by the two methods. There was no statistically significant difference between the mutation frequencies of plasma samples detected with PAP and ARMS PCR ($p = 0.069$). This indicated that the detection of the plasma *EGFR* through the PAP technology appears to be an alternative choice with relatively high sensitivity. However, 9 cases (10.6%) of discordant results were found through the two methods, including both wild-type status and mutant abundance. Among them, two cases (22.2%) of PAP results were correctly identified according to the tumor tissue results. While for ARMS, 7 cases (77.8%) were consistent with tissue status. Thus, for these discordant results, PAP showed a relatively lower sensitivity than ARMS in detecting plasma *EGFR* mutations which might be caused by its bypass reaction [14]. Since PAP appeared to be a reliable method, further analysis was performed to detect whether there was a positive correlation between the *EGFR* mutation load in plasma by this method and the characteristics. A positive correlation between mutation load and gender was observed, indicating that mutation status was more likely to be detected in female patients by PAP, which was consistent with previous studies [20,21]. In clinical practice, PAP may be recommended to be a supplement for patients, especially female patients, when wild-type status is identified by another method.

As the standard method, ARMS is utilized as clinical routine in our center while both NGS and ddPCR are not commonly utilized. We selected ARMS, which could detect mutations in tissue samples containing as little as 1% mutated DNA [22], for this comparative study. It was reported that an ARMS-based method, so-called ARMS-Plus, had been established and proven to be a highly sensitive assay in clinical practice [23]. However, the best method to detect plasma *EGFR* mutations still remains to be explored. The appropriate methods may vary according to specific mutation status. T790M mutation, which is critical for target therapy, is continuously a concern. To detect *EGFR* T790M mutation, ddPCR assay has been proven to be more sensitive than ARMS [24]. This view was supported by similar studies. Recently, it was reported that T790M mutation was only identified by NGS and ddPCR but not ARMS, indicating a disadvantage of ARMS for this resistance mutation [25]. In this study, only 7 cases carried T790M mutation identified by ARMS. This resistance mutation was also identified by PAP. Limited to a small number of cases, no further analysis was performed. However, we still believe that it is worth evaluating PAP or other methods in testing specific mutation status in further investigation with a larger cohort of patients.

CONCLUSION

EGFR mutations including 19Del, L858R, and T790M could be identified by PAP in cfDNA testing. The investigation results showed that there was no statistically significant difference in the clinical performances between PAP and ARMS. Our findings indicate that PAP is a reliable and cost-effective method for the detection of plasma *EGFR* mutations.

Funding:
This study was supported by grants from the Major projects of the Tianjin Municipal Science and Technology Commission (grant 18ZXDBSY00050); Tianjin science and Technology Commission (grant 13JCQNJC 23600); Science and Technology fund of Tianjin Health Bureau (grant 2015KZ085) and the Clinical Trial Foundation of Tianjin Tumor Hospital (grant C1712).

Declaration of Interest:
The authors have no conflicts of interest to declare.

Ethical Statement:
The study was approved by the Tianjin Medical University Cancer Institute and Hospital (Ethical number E201 8086) and written informed consent was obtained from all patients.

References:

1. Gerber DE, Paik PK, Dowlati A. Beyond adenocarcinoma: current treatments and future directions for squamous, small cell, and rare lung cancer histologies. Am Soc Clin Oncol Educ Book 2015:147-62 (PMID: 25993153).

2. Ferlay J, Soerjomataram I, Dikshit R, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015;136:E359-86 (PMID: 2522 0842).

3. Heist RS, Engelman JA. SnapShot: non-small cell lung cancer. Cancer cell 2012;21:448.e442 (PMID: 22439939).

4. Herbst RS, Heymach JV, Lippman SM. Lung cancer. N Engl J Med 2008;359:1367-80 (PMID: 18815398).

5. Shi Y, Li J, Zhang S, et al. Molecular Epidemiology of *EGFR* Mutations in Asian Patients with Advanced Non-Small- Cell Lung Cancer of Adenocarcinoma Histology - Mainland China Subset Analysis of the PIONEER study. PLoS One 2015;10: e0143515 (PMID: 26599344).

6. Yang JC, Wu YL, et al. Epidermal growth factor receptor mutation analysis in previously unanalyzed histology samples and cytology samples from the phase III Iressa Pan-ASia Study (IPASS). Lung Cancer 2014;83:174-81 (PMID: 24361280).

7. Pirker R, Pereira JR, von Pawel J, et al. *EGFR* expression as a predictor of survival for first-line chemotherapy plus cetuximab in patients with advanced non-small-cell lung cancer: analysis of data from the phase 3 FLEX study. Lancet Oncol 2012;13:33-42 (PMID: 22056021).

8. Thierry AR, El Messaoudi S, Gahan PB, Anker P, Stroun M. Origins, structures, and functions of circulating DNA in oncology. Cancer Metastasis Rev 2016;35:347-76 (PMID: 27392603).

9. Mayo-de-Las-Casas C, Jordana-Ariza N, Garzon-Ibanez M, et al. Large scale, prospective screening of *EGFR* mutations in the blood of advanced NSCLC patients to guide treatment decisions. Ann Oncol 2017;28:2248-55 (PMID: 28911086).

10. Li YS, Jiang BY, Yang JJ, et al. Unique genetic profiles from cerebrospinal fluid cell-free DNA in leptomeningeal metastases of *EGFR*-mutant non-small-cell lung cancer: a new medium of liquid biopsy. Ann Oncol 2018;29:945-2 (PMID: 29346604).

11. Helman E, Nguyen M, Karlovich CA, et al. Cell-Free DNA Next-Generation Sequencing Prediction of Response and Resistance to Third-Generation *EGFR* Inhibitor. Clin Lung Cancer 2018;19: 518-30 (PMID: 30279111).

12. Luo J, Shen L, Zheng D. Diagnostic value of circulating free DNA for the detection of EGFR mutation status in NSCLC: a systematic review and meta-analysis. Sci Rep 2014;4:6269 (PMID: 25201768).

13. Kim E, Feldman R, Wistuba II. Update on EGFR Mutational Testing and the Potential of Noninvasive Liquid Biopsy in Non-Small-cell Lung Cancer. Clin Lung Cancer 2018;19:105-14 (PMID: 28935493).

14. Liu Q, Sommer SS. PAP: detection of ultra rare mutations depends on P* oligonucleotides: "sleeping beauties" awakened by the kiss of pyrophosphorolysis. Hum Mutat 2004;23:426-36 (PMID:15108273).

15. Yu HA, Arcila ME, Rekhtman N, et al. Analysis of tumor specimens at the time of acquired resistance to *EGFR*-TKI therapy in 155 patients with *EGFR*-mutant lung cancers. Clin Cancer Res 2013;19:2240-7 (PMID: 23470965).

16. Asamura H, Chansky K, Crowley J, et al. The International Association for the Study of Lung Cancer Lung Cancer Staging Project: Proposals for the Revision of the N Descriptors in the Forthcoming 8th Edition of the TNM Classification for Lung Cancer. J Thorac Oncol 2015;10:1675-84 (PMID: 26709477).

17. Kwak E. The role of irreversible HER family inhibition in the treatment of patients with non-small cell lung cancer. Oncologist 2011;16:1498-507 (PMID: 22016476).

18. Shi J, Liu Q, Sommer SS. Detection of ultrarare somatic mutation in the human TP53 gene by bidirectional pyrophosphorolysis-activated polymerization allele-specific amplification. Hum Mutat 2007;28:131-6 (PMID: 17041903).

19. Chen Z, Feng J, Buzin CH, et al. Analysis of cancer mutation signatures in blood by a novel ultra-sensitive assay: monitoring of therapy or recurrence in non-metastatic breast cancer. PLoS One 2009;4: e7220 (PMID: 19789704).

20. Fukuoka M, Yano S, Giaccone G, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (The IDEAL 1 Trial) (corrected). J Clin Oncol 2003;21:2237-46 (PMID: 12748244).

21. Kris MG, Natale RB, Herbst RS, et al. Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. JAMA 2003;290:2149-58 (PMID: 14570950).

22. Fukuoka M, Wu YL, Thongprasert S, Sunpaweravong P, et al. Biomarker analyses and final overall survival results from a phase III, randomized, open-label, first-line study of gefitinib versus carboplatin/paclitaxel in clinically selected patients with advanced non-small-cell lung cancer in Asia (IPASS). J Clin Oncol 2011;29:2866-74 (PMID: 21670455).

23. Wang L, Guo Q, Yu W, et al. Quantification of plasma *EGFR* mutations in patients with lung cancers: Comparison of the performance of ARMS-Plus and droplet digital PCR. Lung Cancer 2017;114:31-7 (PMID: 29173762).

24. Wang W, Song Z, Zhang Y. A Comparison of ddPCR and ARMS for detecting EGFR T790M status in ctDNA from advanced NSCLC patients with acquired EGFR-TKI resistance. Cancer Med 2017;6:154-62 (PMID: 28000387).

25. Li Y, Lv J, Wan S, et al. High Sensitive and Non-invasive ctDNAs Sequencing Facilitate Clinical Diagnosis And Clinical Guidance of Non-small Cell Lung Cancer Patient: A Time Course Study. Front Oncol 2018;8:491 (PMID: 30425968).