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Next-generation sequencing-based detection of *EGFR*, *KRAS*, *BRAF*, *NRAS*, *PIK3CA*, *Her-2* and *TP53* mutations in patients with non-small cell lung cancer

CHANGWEN JING[1*], XUHUA MAO[2*], ZHUO WANG[1*], KEJING SUN[3], RONG MA[1], JIANZHONG WU[1] and HAIXIA CAO[1]

[1]Clinical Cancer Research Center, Jiangsu Cancer Hospital and Cancer Institute of Jiangsu Province and The Affiliated Cancer Hospital of Nanjing Medical University, Nanjing, Jiangsu 210009; [2]Clinical Laboratory, Yixing People's Hospital, Yixing, Jiangsu 214200; [3]Genesmile Company, Nanjing, Jiangsu 210009, P.R. China

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Abstract. In recent years, the incidence of non-small cell lung cancer (NSCLC) has become the highest lethal rate of cancer worldwide. Molecular assays of *EGFR*, *KRAS*, *BRAF*, *NRAS*, *PIK3CA* and *Her-2* are widely used to guide individualized treatment in NSCLC patients. Somatic mutations in 112 NSCLC patients, including 7 oncogenic driver genes, were detected by Iontorrent personal genome machine (PGM). Sanger sequencing was used to test and verify the results of PGM. Apart from uncommon mutations of *EGFR*, 101 NSCLC specimens were tested by droplet digital PCR (ddPCR). According to NGS results, mutations were detected in *EGFR* (58/112, 51.79% of tumors), *KRAS* (10/112, 8.93%), *BRAF* (2/112, 1.79%), *NRAS* (2/112, 1.79%), *Her-2* (2/112, 1.79%), *PIK3CA* (6/112, 5.36%) and *TP53* (31/112, 27.69%). There were 27 samples without any somatic mutations in all genes while 24 samples harboured mutations in two or more genes. A total of 61 samples had one or more mutations in a single gene. All alterations of 7 genes were presented and the overall detection rate of NGS and Sanger sequencing was determined to be 51.79% (58/112) and 37.50% (42/112), respectively (χ^2=5.88, P=0.015). Compared with Sanger sequencing, the total sensitivity and specificity of NGS assays was 95.24% (40/42) and 77.14% (54/70), respectively. The overall detection rate of NGS and ddPCR was 45.54% (46/101) and 47.52% (48/101), respectively (χ^2=0.000598, P=0.98). Compared with ddPCR, the overall sensitivity and specificity of NGS assays was 95.83% (46/48) and 98.11% (52/53), respectively. The findings indicated that the positive mutation rate of EGFR tested by NGS was significantly lower than that by Sanger sequencing, but the difference between ddPCR and NGS was not statistically significant. The high degree of agreement of reportable variants is proposed in both NGS and ddPCR analysis, suggesting the performance of NGS assays in routine clinical detection may be useful in determining the treatment decisions in NSCLC patients.

Correspondence to: Mr. Jianzhong Wu or Ms. Haixia Cao, Clinical Cancer Research Center, Jiangsu Cancer Hospital and Cancer Institute of Jiangsu Province and The Affiliated Cancer Hospital of Nanjing Medical University, 42 Baiziting Road, Nanjing, Jiangsu 210009, P.R. China
E-mail: wujzh1528@126.com
E-mail: pascallschx@126.com

[*]Contributed equally

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Introduction

Non-small cell lung cancer (NSCLC) is the most common histological type of lung cancer, accounting for about 80-85% of total lung cancer. In recent years, the incidence of NSCLC continues to increase and has become the highest lethal rate of cancer all over the world (1). The application of epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs), such as gefitinib, has improved the treatment of NSCLC (2). Detection of sensitive mutations to EGFR-TKIs has stimulated the interest in studying multiple oncogenic drivers. Previous results suggested that somatic mutations in *EGFR*, *KRAS*, *BRAF*, *NRAS*, *PIK3CA*, *Her-2* and *TP53* have been associated with efficacy of EGFR-TKIs, metastasis or overall survival (3-6). Therefore, molecular assays of *EGFR*, *KRAS*, *BRAF*, *NRAS*, *PIK3CA* and *Her-2* are widely used to guide individualized treatment in NSCLC patients.

Commonly used technologies for oncogenic driver detection include direct sequencing, next-generation sequencing (NGS), amplification refractory mutation system (ARMS) and droplet digital PCR (ddPCR). Sanger sequencing is used as standard for detecting EGFR mutations because of accurate results and low throughput. However, it is limited by high cost, time consuming and low sensitivity, for detecting low frequency mutant alleles in a specimen mixed with normal alleles. ddPCR is a new generation of absolute quantification PCR technique, realizing the independent amplification and

fluorescence reading of thousands of individual droplets in one well. It has an extremely high sensitivity (0.04%-0.1%) and each well can only detect one site, limiting its use in multiple assays (7). Next Generation Sequencing (NGS) is a method that can detect multiple genetic variations simultaneously and can detect tumor mutations efficiently and economically. The scientists had a blinded comparison of NGS and quantitative real-time PCR (qPCR) assays to detect mutations in EGFR, KRAS, PIK3CA and BRAF in Chinese patients with NSCLC. Sanger sequencing was used to verify the inconsistent results of qPCR and NGS assays. The high consistency between NGS and qPCR has shown clinical application prospects of NGS (8).

In the present study, we detect somatic mutations in NSCLC by a small panel including 7 genes using the Iontorrent personal genome machine (PGM), to evaluate the efficacy of NGS by comparison to ddPCR assay and Sanger sequencing.

Patients and methods

Patient characteristics. Non-small lung tumor tissues were obtained from 112 Chinese patients in Jiangsu Cancer Hospital (Nanjing, China) between June 2015 and June 2016. Clinical characteristics of all patients were recorded with detailed information summarized in Table I. The histological diagnosis of all samples was confirmed by the pathologists. TNM classification of malignant tumors was used to determine tumor stage. All patients participated in the study signed informed consent. The ethics approval was awarded by the Cancer Institute of Jiangsu Province Ethics Committee.

DNA extraction from formalin-fixed paraffin-embedded (FFPE) tissue. Tumor-rich samples were obtained when the patient underwent surgery. DNA was extracted with DNA FFPE tissue kit (Omega, Norcross, GA, USA) according to the guidebook and the concentrations were detected by Qubit® 2.0 fluorometer dsDNA HS assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Mutation analysis by NGS. The Lung panel (including *BRAF, EGFR, KRAS, NRAS, PIK3CA, Her-2* and *TP53*) on Iontorrent system was generally provided by Thermo Fisher Scientific, Inc. DNA was extracted and purified after microdissection using Agencourt® AMPure™ XP beads (Beckman Coulter, Brea, CA, USA). After DNA concentration detection, 15 ng of DNA was then amplified, fragmented, ligated to adapters, barcoded, and clonally amplified onto beads to create DNA libraries, using Iontorrent ampliSeq kit 2.0 and IonXpress barcode adapters kit (Thermo Fisher Scientific, Inc.) by user guidebook. After quantification, library mixtures were amplified with Iontorrent Onetouch template kit (Thermo Fisher Scientific, Inc.) and enriched on Iontorrent Onetouch system according to the protocol. Finally, the library pool was sequenced with Iontorrent PGM sequencing supplies 200 v2 kit (Thermo Fisher Scientific, Inc.) using Iontorrent PGM system. The mutation site was analyzed by the Iontorrent variant caller plugin v4.0 according to the reference genome hg19. The threshold of mutation frequency for mutation was 1%. The overall median coverage of depth was >1000X. The sequencing coverage of amplicons is >1,000 and the uniformity was >90%.

Table I. Patient characteristics (n=112).

Variables	Number of patients
Sex	
Male	67
Female	45
Age	
<60 years	35
≥60 years	77
Histological type	
Adenocarcinoma	82
Squamous cell carcinoma	24
Adenosquamous carcinoma	1
Others	5
Histopathological grading	
High-median	33
Low	79
TNM staging	
I-II	34
III-IV	78

Sanger sequencing. Firstly, PCR was performed in a PCR Amplifier (Biometra, GmbH, Göttingen, Germany) for Sanger sequencing. Primers used for exon 18-21 of EGFR were listed in Table II. Secondly, PCR products were purified by Axyprep™ PCR cleanup kit (Axygen, Hangzhou, China). Thirdly, sequencing reaction was performed with big dye terminator v3.1 (Thermo Fisher Scientific, Inc.). Finally, the products were denatured and analysed by a DNA sequencer (Applied Biosystems 3500; Thermo Fisher Scientific, Inc.). Sequencing analysis v5.4 software was used to analyze the results.

Droplet digtal PCR. Genotypes with L858R, exon 19 deletion, T790M or G719S were conducted by ddPCR. 20 μl of PCR reaction mixtures were prepared. After droplets generation, the products were shifted to a 96-well plate for amplification. The amplified products were analyzed on QX200™ Droplet Digital™ PCR (BioRad Laboratories, Inc., Hercules, CA, USA). The samples which contained at least 2 droplets in the FAM positive area were called positive.

Statistical analysis. The ability of NGS and ddPCR platforms to detect EGFR mutations was analyzed using χ^2 test with IBM SPSS Statistics for Windows (v19.0. IBM Corp., Armonk, NY, USA). P<0.05 represents statistically significant differences. All figures were produced with GraphPad Prism (v6.0; GraphPad Software, Inc., La Jolla, CA, USA).

Results

The patient mutation profile. There were 86 FFPE specimens, 26 fresh resection specimens, 13 fine needle aspiration specimens and 4 pleural effusion specimens. Finally, 17 specimens failed to pass quality control. The remaining

Table II. Primers for direct sequencing.

Exon	Primer name	Sequence
18	EGFR 18S F	5'-AGCATGGTGAGGGCTGAGGTGAC-3'
	EGFR 18S R	5'-ATATACAGCTTGCAAGGACTCTGG-3'
19	EGFR 19S F	5'-CCAGATCACTGGGCAGCATGTGGCACC-3'
	EGFR 19S R	5'-AGCAGGGTCTAGAGCAGAGCAGCTGCC-3
20	EGFR 20S F	5'-GATCGCATTCATGCGTCTTCACC-3'
	EGFR 20S R	5'-TTGCTATCCCAGGAGCGCAGACC-3'
21	EGFR 21S F	5'-TCAGAGCCTGGCATGAACATGACCCTG-3'
	EGFR 21S R	5'-GGTCCCTGGTGTCAGGAAAATGCTGG-3'

F, Forward; R, Reverse; EGFR, epidermal growth factor receptor.

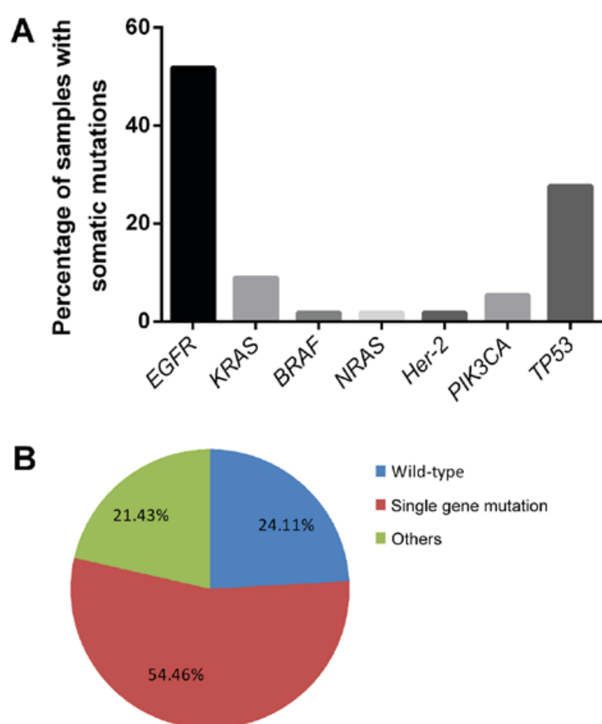

Figure 1. (A) Incidences of *EGFR*, *KRAS*, *BRAF*, *NRAS*, *PIK3CA* and TP53 mutations detected by NGS. (B) Proportion of patients carrying wild-type, one gene and two or more gene mutations. NGS, next-generation sequencing.

112 specimens were successful submitted to NGS. As shown in Fig. 1A, mutations were detected in *EGFR* (58/112, 51.79% of tumors), *KRAS* (10/112, 8.93%), *BRAF* (2/112, 1.79%), *NRAS* (2/112, 1.79%), *Her-2* (2/112, 1.79%), *PIK3CA* (6/112, 5.36%) and *TP53* (31/112, 27.69%). Fig. 1B showed that there were 27 samples without any somatic mutations in all genes while 24 samples harboured mutations in two or more genes. 61 samples had mutations in single gene. Concomitant *EGFR* and *TP53* mutations accounted for 54.17% (13/24) of samples with multiply gene mutations including two specimens with triple gene alterations (*EGFR*, *KRAS*, *TP53*/*EGFR*, *PIK3CA*, *TP53*). There were 3 samples occupied *KRAS* and *TP53* mutations. Concomitant *TP53* and *PIK3CA* mutations occurred in 2 NSCLC patients. Doublet mutations of *EGFR* and *PIK3CA*, *EGFR* and *KRAS*, *EGFR* and *NRAS*, *EGFR* and *Her-2*, *BRAF* and *TP53*, *BRAF* and *PIK3CA* occurred in 1 NSCLC each.

Genetic alterations of 7 genes. *EGFR* mutations. All genetic alterations of *EGFR* gene were illustrated in Table III. Mutations were found in 6 samples in exon 18, 29 in exon 19 including 21 samples of 19 deletions, 2 in exon 20 and 34 in exon 21. There are 56 cases with *EGFR* mutations in adenocarcinoma and two in squamous cell carcinoma. Ten samples have doublet mutations in *EGFR* gene. The distribution of doublet *EGFR* mutations was one with L858R and E746_S752del, one with L858R and E746_A750del, one with C781S and E746_T751delinsE, one with L858R and V834L, one with L747_P753delinsS and T790M, one with E746_R748del and A750P, one with E746_R748del and K754E, one with E749Q and A750P and two with G719S and E709K. One sample harboured quadruple mutations with L861Q, L858R, E745_A750del and G729A.

KRAS mutations. *KRAS*, *BRAF*, *Her-2*, *NRAS* and *PIK3CA* mutations were shown in Table IV. Representative types of genetic alterations in *KRAS* were six, with all of these located in exon 2. One sample harboured doublet *KRAS* mutations with G12C and G12A.

BRAF mutations. Two samples carried *BRAF* mutations were p.V600E and p.L618F.

NRAS mutations. One patient carried *NRAS* D33E mutations plus EGFR 19 deletion while another patient carried *NRAS* G12D alteration.

Her-2 mutations. Both of the patients with *Her-2* mutations were P761H mutation.

PIK3CA mutations. There were 6 cases of *PIK3CA* mutations, with 4 located in exon 9 and 2 in exon 20. One sample carried doublet *PIK3CA* mutations with E545K and E542K. The remaining patients with *PIK3CA* mutations all have other gene mutations such as *BRAF*, *EGFR* or *TP53*.

TP53 mutations. The tumour suppressor gene *TP53* mutations are diverse. 25 classes of mutations occurred in *Tp53*.

Comparison of NGS, Sanger sequencing and ddPCR for detecting EGFR mutations. Sanger sequencing, as golden standard, were performed all 112 specimens. The overall detection

Table III. *EGFR* mutations detected by NGS.

Exon	*EGFR* mutation site	Protein position	Number of mutations
Exon 18	c.2155G>A	p.G719S	3
	c.2125G>A	p.E709K	2
	c.2127_2129del	p.E709_T710delinsD	1
Exon 19	c.2245G>C	p.E749Q	1
	c.2248G>C	p.A750P	5
	c.2260A>G	p.K754E	1
	c.2186G>C	p.G729A	1
	c.2238_2252del	p.E746_T751delinsE	3
	c.2236_2244del	p.E746_R748del	2
	c.2239_2256del	p.L747_S752del	1
	c.2236_2250del	p.E746_A750del	3
	c.2236_2256del	p.E746_S752del	1
	c.2235_2249del	p.E746_A750del	5
	c.2236_2249del	p.E746_A750del	1
	c.2237_2251del	p.E746fs	1
	c.2240_2257del	p.L747_P753delinsS	4
Exon 20	c.2369C>T	p.T790M	1
	c.2341T>A	p.C781S	1
Exon 21	c.2573T>G	p.L858R	30
	c.2471G>C	p.G824A	1
	c.2582T>A	p.L861Q	1
	c.2588G>A	p.G863D	1
	c.2500G>T	p.V834L	1

NGS, next-generation sequencing.

rate of NGS and Sanger sequencing was 51.79% (58/112) and 37.50% (42/112), respectively (χ^2=5.88, P=0.015). There were 18 samples owning low frequency of mutations according to NGS results. In 58 positive samples, 40 samples were identified both by NGS and Sanger sequencing. 16 mutation-positive samples in NGS results became negative by Sanger sequencing and two negative samples were identified as positive by Sanger sequencing (Table V). Compared to Sanger sequencing, the total sensitivity and specificity of NGS assays was 95.24% (40/42) and 77.14% (54/70), respectively. Fig. 2 showed that rare mutations with 19 deletion and E-20 c.2341T>A mutation were also found in Sanger sequencing. Apart from uncommon mutations of *EGFR*, ddPCR was conducted in 101 NSCLC specimens. As shown in Table VI, the overall positive rate of NGS and ddPCR was 45.54% (46/101) and 47.52% (48/101), respectively (χ^2=0.000598, P=0.98). Compared to ddPCR, the overall sensitivity and specificity of NGS assays was 95.83% (46/48) and 98.11% (52/53), respectively. The advantages and disadvantages of the NGS, Sanger and ddPCR were presented in Table VII.

Discussion

In lung cancer, the mutational status of oncogenic driver can implicate the efficacy of EGFR-TKIs and future survival for patients. It has been reported that EGFR mutation status resulted in the structural changes in the tyrosine kinase domain of EGFR. The main types of *EGFR* mutations were in exon19 deletions and exon21 (L858R) (9). Consistent with previous research (10), more EGFR mutations were detected in adenocarcinomas compared with squamous cell carcinoma. The positive rate of *EGFR* was 68.29% (56/82) in adenocarcinoma vs. 8.33% (2/24) in squamous cell carcinoma. The patients with *KRAS* mutations may not respond to EGFR antibodies and EGFR kinase inhibitors (11). A study in 5125 samples from NSCLC patients revealed that 8.0% of *KRAS* mutations were located in exon 2 and exon 3 (12). In this investigation, all genetic alterations in *KRAS* were found in exon 2. The quantity of samples attributed to the different results. Moreover, additional targeted drugs for NSCLC patients include *BRAF* inhibitors. Melanomas with *BRAF* mutations have been reported to be highly sensitive to *BRAF* inhibitors (13). Dabrafenib, a *BRAF* inhibitor is currently undergoing phase 2 trial for treatment of V600E *BRAF*-mutant lung adenocarcinomas, which may become another new drug in individualized therapy for lung cancer patients. *BRAF* V600E mutated lung cancer is a genetically distinct subtype that occurs in 1.7% of non-small cell lung carcinomas and 2.3% among 646 adenocarcinomas (14). However, we found only 1 sample (1/112) with p.V600E and 1 with p.L618F. One of the BRAF-positive samples was also *PIK3CA*-mutated, and one had a *TP53* mutation. HER2, a member of the human

Table IV. *KRAS*, *BRAF*, *Her-2*, *NRAS* and *PIK3CA* mutations detected by NGS sequencing.

Gene	Mutation site	Protein position	Number of mutations
KRAS	c.34G>T	p.G12C	2
	c.37G>T	p.G13C	1
	c.35G>A	p.G12D	4
	c.34G>A	p.G12C	1
	c.35G>T	p.G12V	2
	c.35G>C	p.G12A	1
BRAF	c.1799T>A	p.V600E	1
	c.1854G>T	p.L618F	1
Her-2	c.2282C>A	p.P761H	2
NRAS	c.99T>G	p.D33E	1
	c.35G>A	p.G12D	1
PIK3CA	c.1624G>A	p.E542K	2
	c.1633G>A	p.E545K	3
	c.3140A>T	p.H1047L	2
TP53	c.338G>T	p.G113V	2
	c.128G>A	p.R43H	2
	c.422G>T	p.R141L	1
	c.337G>T	p.G113C	1
	c.431C>G	p.A144G	2
	c.105delG	p.Q35fs	1
	c.437C>A	p.P146H	1
	c.443G>C	p.R148T	1
	c.329G>A	p.C110Y	1
	c.335G>A	p.G112D	2
	c.422G>A	p.R141H	1
	c.98C>G	p.P33R	5
	c.415G>T	p.E139X	1
	c.326C>T	p.S109F	1
	c.448C>T	p.R150W	1
	c.320delA	p.N107fs	1
	c.422G>C	p.R141P	1
	c.401G>T	p.G134V	2
	c.353 C>T	p.P118L	1
	c.419_439 del	p.140_147 del	1
	c.436 C>T	p.P146S	1
	c.326 C>A	p.S109Y	1
	c.305 A>C	p.Y102S	1
	c.428 G>T	p.C143F	1
	c.424 G>T	p.V142F	1

NGS, next-generation sequencing.

Table V. Performance of NGS and Sanger sequencing platforms for detection of epidermal growth factor receptor mutation.

	Sanger sequencing (+)	Sanger sequencing (-)
NGS (+)	40	16
NGS (-)	2	54

NGS, next-generation sequencing.

Table VI. Performance of NGS and ddPCR platforms for detection of EGFR mutation.

Variable	ddPCR (+)	ddPCR (-)
NGS (+)	46	1
NGS (-)	2	52

NGS, next-generation sequencing; ddPCR, droplet digital polymerase chain reaction.

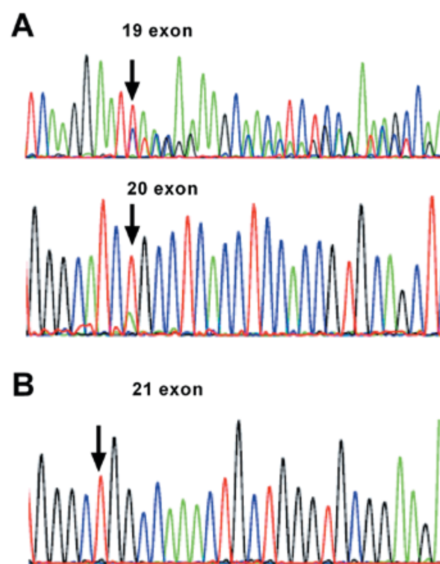

Figure 2. (A) One example containing exon 20 mutation (genotype, E-20 c.2341T>A) and exon 19 deletion (genotype, 2240-2254 deletion) that was verified by Sanger sequencing. (B) A detectable mutation in exon 21 (genotype, 2573T>G) tested by NGS was negative through Sanger sequencing. NGS, next-generation sequencing.

EGFR (ErbB) family, is a receptor tyrosine kinase is encoded by the *Her-2* gene. It is involved in PI3K-Akt and MEK/ERK signaling pathways, associated with cell proliferation and migration (15). *Her-2* mutations have been found in 2-4% of lung adenocarcinomas (16,17). The frequency of *Her-2* mutation was 1.8% in our present studies, all in exon 19 mutations not exon 20. The results are different from the previous reports, probably due to geographical area and sample size.

Our investigation found 2 patients with three types of gene mutations and 22 patients with two types. It is rare that mutations in *NRAS* and *KRAS* occur along with other driver-driven genetic alterations. Although concomitant mutations of some double genes seem paradox theoretically, we found TKI-sensitive and TKI-resistant variants co-existed. Overlap mutations in driver genes may puzzle clinical doctors in making individualized treatment for lung cancer. The sensitivity analysis of these patients to EGFR-TKIs requires follow-up. Advanced lung cancer patients with *EGFR*

Table VII. Characteristics of the NGS, Sanger sequencing and ddPCR.

Features	Sanger sequencing	ddPCR	NGS
Frequency quantity	No	Yes	Yes
Sensitivity	10%	0.04-0.1%	0.1%
Coverage area	Common/uncommon mutations	Common mutations	Common/uncommon mutations
Time for results	2-3 days	1 day	2-3 days
Technical characteristics	High accuracy, only suitable for tissues	Very high sensitivity but questioned by false-positive error	High output

NGS, next-generation sequencing; ddPCR, droplet digital polymerase chain reaction.

mutations or *KRAS* mutations and *PIK3CA* mutations have a poor prognosis. Patients with concurrent *PIK3CA* and *EGFR* mutations can not benefit from EGFR-TKIs (18). One study reported the mutation characteristics of patients with stage 1b lung adenocarcinoma in China. The results showed that only one patient had *EGFR* T790M mutation and *KRAS* mutation. No other *EGFR* mutation coexisting with *KRAS* was found (19). We believe there will be more reports of concurrent mutations in driver genes in the future, and the clinical detection of multiple oncogene mutations can help determine the optimal treatment regimen.

High throughput sequencing has not only provided us with rich genetic information, but also greatly reduced the cost and time of sequencing, with high output and high resolution. This technology has been applied wildly in cancer research. Previous reports have shown that though the frequency of single gene mutations in lung cancer may be low, the mutation rate of multiple oncogenic driver genes was really high. Individuals with oncogenic driver gene mutations receiving targeted therapy lived longer (20). In this study, we carried out NGS in NSCLC patients to evaluate the efficacy of NGS by comparison to ddPCR assay and Sanger sequencing. Our results showed NGS-based methods have demonstrated performance sensitivity but low specificity of NGS due to 18 low frequency mutant specimens compared to Sanger sequencing. Among them, 16 specimens were *EGFR* wild-type by Sanger sequencing. In addition, the results of NGS and ddPCR test were highly consistent. The high clinical sensitivity and specificity support the routine use of NGS detection in clinical trials to promote the treatment of patients with lung cancer. The detection rate of NGS for *EGFR* was significantly higher than that of Sanger sequencing. However, there was no significant statistical difference between ddPCR and NGS results. Besides, NGS can detect both hotspot and non-hotspot mutations. In general, ddPCR diagnostic kits are commonly used to detect common mutations or hotspots. However, rare mutations in EGFR are also important for predicting the efficacy of EGFR-TKI drugs, so the identification of non-hotspot mutations is essential for clinical research and treatment (2,21). In EMSO 2017, AURA17 studies initiated by Zhou *et al* (22) demonstrated the objective response rates (ORR) of ochitinib in patients with T790M mutations detected by the three detection methods were 56% (Cobas; Roche Diagnostics, Basel, Switzerland), 64% (SuperARMS) and 56% ddPCR, respectively. Furthermore, ORR of ochitinib in patients without T790M mutations detected by Cobas and ddPCR was higher than that in positive patients. Whether there is false-negative and false-positive error made by ddPCR is also needed for further study. However, NGS has its limitation. In the process of high-throughput sequencing, there are many problems that need to be solved: the role of data in clinical diagnosis, storage and analysis of sequencing data, data security and information privacy.

In conclusion, our results show that NGS has the advantages of high sensitivity and multiplexed testing. More data should be required to evaluate sensitivity, stability and clinical applicability. Each detection method has its advantages and disadvantages. Practice is the sole criterion for testing truth, and the benefit of cancer patients after treatment is the only criterion for judging methods. Detection methods should complement each other to achieve balance and coexistence, maximizing benefit of patients. In daily work, Sanger sequencing and ddPCR, as supplement of NGS results, are suggested to confirm uncommon mutations and low frequency mutations, respectively.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CJ, XM and ZW wrote the manuscript and designed the study. KS performed the ddCPR experiments. RM performed NGS and Sanger sequencing. JW and HC contributed to the design of the study. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The research using human tissue was approved by the Cancer Institute of Jiangsu Province Ethics Committee. All patients participated in the study signed informed consent.

Patient consent for publication

All patients participated in the study signed informed consent.

Competing interests

The authors declare that they have no competing interests.

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