Original Article

Comprehensive analysis of next generation sequencing and ARMS-PCR for detecting EGFR exon 20 insertion (ex20ins) mutations in Chinese non-small cell lung cancer patients

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Background: Amivantamab (JNJ-372) and mobocertinib (TAK-788) have been reported to have favorable therapeutic effect for non-small cell lung cancer (NSCLC) patients with epidermal growth factor receptor (EGFR) exon 20 insertion (ex20ins) mutations. Thus, accurate detection of *EGFR ex20ins* mutations is crucial for subsequent individualized therapy. The aim of this study was to compare the two common methods of next generation sequencing (NGS) and amplification refractory mutation system polymerase chain reaction (ARMS-PCR) for detecting *EGFR ex20ins* mutations in Chinese NSCLC patients.

Methods: We retrospectively analyzed EGFR mutations, especially for ex20ins, in 3,606 NSCLC patients detected by NGS and 1,785 patients by ARMS.

Results: Among the 3,606 NGS patients, a total of 2,077 EGFR mutations and 95 *EGFR ex20ins* were identified, accounting for 57.6% and 2.6%, respectively. While 48.4% of EGFR mutations and 1.1% of ex20ins were detected in 1,785 ARMS patients, which were significantly lower than those of NGS (P<0.01). Thirty-four unique ex20ins variants were identified by NGS, and eight of them was reported for the first time. However, ARMS was designed to detect only several known *EGFR ex20ins* variants, and even did not include the most common variants in Chinese NSCLC patients.

Conclusions: NGS is more advantageous and strongly recommended for the detection of *EGFR ex20ins* mutations. Considering the fast and cost-effective ARMS detection method, it is suggested that the primers design should be updated according to the characteristics of *EGFR ex20ins* mutations in Chinese NSCLC patients.

Keywords: Non-small cell lung cancer (NSCLC); epidermal growth factor receptor exon 20 insertion (*EGFR ex20ins*); next generation sequencing (NGS); amplification refractory mutation system (ARMS)

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Introduction

Lung cancer is a leading cause of cancer incidence and mortality with an estimated 1.2 million diagnosis and 1.8 million deaths per year, globally (1). Non-small cell lung cancer (NSCLC) accounts for 85–90% of the lung cancer cases (2,3). Approximately 30% of NSCLC tumors harbor a mutation in the *EGFR* gene (4), with geographical variation in rates, the frequency of epidermal growth factor receptor (EGFR) genomic alterations was identified as 50.1% in Chinese NSCLC populations and significantly higher than the Western population, which was about 15% (5,6), and predominantly found in female, non-smoking, adenocarcinoma patients (7). *EGFR* tyrosine kinase inhibitors (TKIs) have led to better management and a standard of care for patients with recurrent or metastatic NSCLC that harbor *EGFR* activating mutations, such as *L858R* in *exon 21* and in-frame deletions in *exon 19* (*ex19del*), which are considered as classical mutations and account for almost 85% of observed *EGFR* mutations in NSCLC (8-10). With the wide application of next generation sequencing (NGS) in clinical practice, more and more rare mutations of *EGFR* have been discovered, of which *EGFR exon 20 insertion* (*ex20ins*) mutations constitute about 4–12% of all *EGFR* mutation types, and hence, are the third most common type of *EGFR* mutations in NSCLC, following *ex19del* and *L858R* point mutation (11-13). *EGFR ex20ins* mutations are reported to occur either toward the C terminal of the C-helix or in the loop that immediately follows it, and they lead to a prominent shift of the C-helix and P-loop into the drug-binding pocket, resulting in significant steric hindrance and limiting its binding to the traditional *EGFR*-TKIs (14,15). Thus, contrary to classical *EGFR* mutations that are sensitive to *EGFR*-TKIs, *EGFR ex20ins* mutations are associated with poor responses to approved first and second *EGFR*-TKIs (14). Further, NSCLC patients with *EGFR ex20ins* are known to have poorer prognosis compared to patients with other sensitizing *EGFR* mutations leading to unmet clinical need of newer specific therapeutic options (16). Fortunately, the US Food and Drug Administration (FDA) has approved mobocertinib (*EGFR*-TKI) and the *EGFR-MET* bispecific antibody amivantamab, as targeted treatments for adult patients with locally advanced or metastatic NSCLC with *EGFR ex20ins* in 2021 (17,18). In several clinical trials, the two targeted drugs were associated with clinically meaningful benefit in patients with previously treated *EGFR ex20ins*-positive NSCLC, with a manageable safety profile (19,20). Given its importance for diagnosis and treatment, accurate detection of *EGFR ex20ins* mutations is particularly important.

Several molecular diagnostic methods are currently used for the identification of *EGFR ex20ins* in NSCLC patients, including amplification refractory mutation system (ARMS), droplet digital polymerase chain reaction (ddPCR), sanger sequencing and NGS. Among them, ARMS and NGS are the most common methods recommended to detect *EGFR* mutations in domestic and foreign Clinical Practice Guidelines. ARMS is known to explore only a small number of certain hot spot EGFR mutations, which usually just contain several *EGFR ex20ins* variants. Therascreen (21,22) and Cobas *EGFR* v2 (23) are the two FDA approved PCR methods used for the assessment of *EGFR* mutations. Therascreen can detect three types of *ex20ins*, and Cobas *EGFR* v2 can detect five types of *ex20ins*. However, based on NGS data, so far, studies have identified at least 85 unique *ex20ins* variants in the Chinese population and 102 variants in the US population, with *A767_V769dup* and *S768_D770dup* being the most common ones (24,25). Therefore, the National Comprehensive Cancer Network (NCCN) and European Society of Medical Oncology (ESMO) guidelines currently recommend NGS to assess oncogenic drivers, including *EGFR ex20ins* in NSCLC patients (26,27). However, the efficacy of ARMS and NGS in detecting

Highlight box

Key findings

- In 3,606 non-small cell lung cancer (NSCLC) patients, 95 epidermal growth factor receptor exon 20 insertion (*EGFR ex20ins*) mutations and 34 kinds of ex20ins variants were identified by next generation sequencing (NGS), accounting for 2.6% of NSCLC patients. And eight variants of which were reported for the first time.
- Only 1.1% of *EGFR ex20ins* mutations were identified from 1,785 NSCLC patients by amplification refractory mutation system (ARMS).

What is known and what is new?

- NGS has the advantages of high flux and diverse detection types in detecting *EGFR ex20ins* mutations.
- Compared with NGS, ARMS is fast and cost-effectiveness, but the false negative rate was about 57.9% to 98.9% based on the primers design in different ARMS kits.

What is the implication, and what should change now?

- In clinical practice, if ARMS methods are still applied for EGFR mutation detection, their primers design should be updated according to the characteristics of *EGFR ex20ins* mutations in Chinese NSCLC patients.

EGFR ex20ins mutations is unclear in clinical practice.

Thus, in this study, we systematically analyzed and compared *EGFR* mutations in 3,606 Chinese NSCLC patients detected by NGS and 1,785 patients by ARMS. Further, we predicted the false negative rate of *EGFR ex20ins* mutation detection using different PCR kits in the real world based on the *EGFR ex20ins* variants detected by NGS. We present this article in accordance with the STROBE reporting checklist (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-23-848/rc).

Methods

Patients and samples

A total of 3,606 patients diagnosed with primary or metastatic NSCLC in Fudan University Shanghai Cancer Center during July 2017 and June 2022 were submitted for detection of *EGFR* gene mutations by NGS using a capture-based targeted sequencing panel (Burning Rock Biotech, Guangzhou, China), including all exons in 68 genes (Table S1). And a total of 1,785 NSCLC patients recruited from March 2019 to June 2022 were detected using ADx-ARMS *EGFR* kit (Amoy Diagnostics CO., ltd., Xiamen, China), which can detect 18 variants of *EGFR ex20ins* mutations (*Table 1*). All test materials were formalin-fixed paraffin-embedded (FFPE) tumor samples, including surgical specimens, fine-needle aspirate and cytology specimens. All the samples were evaluated by an experienced pathologist prior to testing, and only samples with tumor cell content higher than 10% were further tested and analyzed. Clinicopathologic features, including patient age, gender, smoking history, pathological types and clinical stage, were obtained from the medical record, pathology report, and/or discharge summary. About 90% of the patients received the NGS and ARMS *EGFR* detection were treatment naïve. The study basically shown the original *EGFR ex20ins* mutation rates. Ethical approval was obtained from the Research Ethics Committee of Fudan University Shanghai Cancer Center (No. 050432-4-2108*). Informed consent was waived due to the retrospective nature of the study. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

DNA-based NGS

Total genomic DNA was extracted from tissue samples using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions. Genomic DNA was profiled by using a capture-based targeted sequencing panel (Burning Rock Biotech), including all exons in 68 genes. The concentration of the DNA samples was measured with the Qubit dsDNA assay (Thermo Fisher Scientific, Waltham, MA, USA). The DNA was then sheered to 300 bp using a Covaris S220 Focused ultrasonicator (Covaris, Woburn, Massachusetts, USA), followed by hybridization with the capture probe baits, hybrid selection with magnetic beads, and PCR amplification. QIAxcel Advanced automated nucleic acid analysis system (Qiagen) was then used to assess the quality and size range. Available indexed samples were then sequenced on a Nextseq 550 System (Illumina, San Diego, CA, USA) with paired-end reads. The sequencing data were mapped to the human genome (hg19) using Burrows-Wheeler Aligner version 0.7.10. Local alignment optimization, variant calling, and annotation were performed using the Genome Analysis Toolkit version 3.2 and VarScan version 2.4.3.

ARMS

Total DNA was extracted from three to four sections of 5μm thick FFPE tissues using a FFPE DNA kit (Qiagen GmbH, Hilden, Germany). The *EGFR* mutation was readily detected using the ARMS commercial regent (Amoy) according to the manufacturer's protocol. The following PCR procedure was used: An initial denaturation at 95 ℃ for 5 min, followed by 95 ℃ for 25 sec, 64 ℃ for 20 sec and 72 ℃ for 20 sec to ensure the specificity, and 31 cycles of 93 ℃ for 25 sec, 60 ℃ for 35 sec and 72 ℃ for 20 sec to perform the data collection.

Statistical analyses

Fisher's exact test was used to compare the gender, age, and smoking history of *EGFR* mutation and *EGFR ex20ins* groups. Chi-squared test for trend was used to analyze the relationship between *EGFR* mutations and pathological type and clinical stage. $P<0.05$ was defined as significant in the analysis.

Results

Clinical characteristics of NSCLC patients with EGFR mutations detected by NGS and ARMS

Among the 3,606 NSCLC patients detected by NGS, 2,077 (57.6%, 2,077/3,606) patients had *EGFR* mutations.

Table 1 Frequencies of different EGFR exon20ins alterations in NSCLC patients detected by NGS and the kinds of EGFR ex20ins mutations that can be detected by different ARMS-PCR kits

Insertion site	NGS mutation	Base changes	Unit mut (n)	ADx-ARMS EGFR kit mutation	Therascreen® EGFR RGQ PCR kit	Roche cobas EGFR Mutation Test V2
Near-loop (AA767-772)	A767_V769dup	2300_2308dupCCAGCGTGG	28	√		√
	S768_D770dup	2303_2311dupGCGTGGACA	15			
	N771_H773dup	2311_2319dupAACCCCCAC	7	√		
	P772_H773dup	2314_2319dup	7			
	D770delinsGY	2308_2309insGTT	3			
	N771dup	2311_2313dup	2			
	V769_D770insGVV	2308_2309insGGGTTGTGG	2			
	D770_N771insG	2310_2311insGGT	1	√	√	√
	D770_N771insGT	2310_2311insGGCACA	1	√		
	N771_P772insT	2313_2314insACC	1	√		
	N771_P772insH	2312_2314dup	1	√		
	P772_H773insQ	2319delinsACAT	1	√		
	D770delinsNNN	2308delinsAACAACA	1			
	S768_V769insVAS	2303_2304insTGTGGCCAG	1			
	D770_N771insGF	2310_2311insGGGTTT	1			
	V769_D770insGSV	2308_2309insGCAGCGTGG	1			
	N771delinsKG	2312_2313insGGG	1			
	N771_P772insRH	2314_2315insGGCACC	1			
	D770_N771insT	2311_2312insCCA	1			
	*D770_N771insST	2311_2312insGCACCA	1			
	*D770_N771insN	2310delinsTAAT	1			
	*V769_D770insCGG	2307_2308insTGTGGGGGG	1			
	*V769delinsCP	2305_2306delinsTGTCC	1			
	*D770delinsANPH	2309_2316delinsCCAACCCTCACAACCCT	1			
	*P772_H773insTNP	2316delinsAACCAACCCT	1			
	*P772_H773insGHP	2316_2317insGGCCACCCC	1			
	V769_D770insASV	2307_2308insGCCAGCGTG	0	√	√	√
	D770_N771insSVE	2311_2312insGCGTCGAAA	0	√		√
	N771_P772insT	2313_2314nsACC	0	√		
	D770_N771insGD	2310_2311insGGGGAC	0	√		
	V769_D770insMASVD	2307_2308insATGGCCAGCGTGGAC	0	√		
	D770_N771insGD	2308_2309insACGGCG	0	√		
	D770_N771insG	2310_2311insGGC	0	√		
	D770_N771insG	2310_2311insGGG	0	√		

Table 1 *(continued)*

Table 1 *(continued)*

Insertion site	NGS Mutation	Base changes	Unit mut (n)	ADx-ARMS EGFR kit mutation	Therascreen® EGFR RGQ PCR kit	Roche cobas EGFR Mutation Test V2
Far-loop (AA773-775)	H773dup	2317_2319dupCAC	4			
	H773_V774insGH	2320_2321insGACACCCCCACG	2			
	H773_V774insAH	2315_2320dup	1			
	H773_V774insPHPH	2319_2320insCCACACCCCCAC	1			
	V774_C775insHV	2316_2321dup	1			
	H773_V774dup	2316_2321dup	1			
	H773_V774insTH	2319_2320insACACAC	1			
	*H773delinsNPY	2317delinsAACCCCT	1			
	H773_V774insH	2319_2320insCAC	0	√	√	√
	H773_V774insQ	2319_2320insCAG	0	√		
	H773_V774insY	2319_2320insTAC	0	√		

√, EGFR mutations detected by ARMS-PCR kits. *, First reported EGFR ex20ins mutations. EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; NGS, next generation sequencing; ARMS, amplification refractory mutation system; PCR, polymerase chain reaction.

Ninety-five patients had *EGFR ex20ins* mutations, contributing to 2.6% of all NSCLC cases (95/3,606) and 4.6% of *EGFR*-mutant tumors (95/2,077). The 3,606 NSCLC patients comprised 1,636 men and 1,970 women, at a median age of 74, ranged from 22–86 years old. The 2,077 patients with *EGFR* mutations comprised 724 men and 1,353 women, at a median age of 61, ranged from 22–86 years old. The 95 *EGFR ex20ins* patients included 33 men and 62 women. The median age was 57 years old, with a range of 22–79 years old. Among the patients with *EGFR* mutation, only 15.9% (330/2,077) patients were ≤50 years old, while the proportion was as high as 46.3% (44/95) in patients with *EGFR ex20ins*, the difference was statistically significant (P<0.001). Most of the NSCLC patients with *EGFR* mutations and/or *EGFR ex20ins* were non-smokers and adenocarcinoma with earlier clinical stage (I and II). There was a significant difference in clinical stages between *EGFR* mutation and *EGFR ex20ins* groups (P=0.05), and *EGFR ex20ins* mutations were more commonly identified in clinical stage I and II. A total of 1,785 NSCLC patients detected *EGFR* mutations by ARMS assay, most of them were in clinical stage III or IV. Eight hundred and sixty-four (48.4%, 864/1,785) patients had *EGFR* mutations, and only 20 patients had *EGFR ex20ins*, contributing to 1.1% of all NSCLC cases (20/1,785) and 2.3% of *EGFR*-mutant tumors (20/864). Similar with the NGS detection group, women, non-smokers, >50 years old and adenocarcinoma patients accounted for the majority of patients with *EGFR* mutations. However, no statistical significance for *EGFR ex20ins* was found among the different clinical stage groups (*Table 2*).

EGFR mutations and ex20ins in NSCLC patients detected by NGS

A total of 2,077 *EGFR* mutations, including 1,850 unique and 227 complex variants, were identified by NGS, accounting for 57.6% of the 3,606 NSCLC patients. In 1,850 *EGFR* unique mutations, *exon 18* mutations (n=44, 2.4%), *exon 19* mutations (n=759, 41.0%), *exon 20* mutations (n=111, 6.0%; ex20ins, n=95, 5.1%), *exon 21* mutations (n=886, 47.9%) and other exon mutations (n=50, 2.7%) were identified. In 227 *EGFR* complex variants, which indicating two to four *EGFR* mutations occurred simultaneously, *exon 20* mutations accounted for 41.9% (95/227), most of them were *T790M* combined with *L858R* or *ex19del*, no complex variants with *EGFR ex20ins* were found (*Figure 1*).

Among the total of 206 *EGFR exon 20* mutation patients, 95 unique *EGFR ex20ins* mutations, 16 unique *exon20* other mutations and 95 complex *exon20* other mutations were identified (*Figure 2*). Of the 95 *EGFR ex20ins* mutations,

Table 2 Summary of clinicopathological characteristics of NSCLC patients with EGFR mutations detected by NGS and ARMS

Characteristics	NGS				ARMS				P value[a]
	Total patients (n=3,606)	EGFR mutation (n=2,077)	EGFR ex 20ins (n=95)	P value	Total patients (n=1,785)	EGFR mutation (n=864)	EGFR ex 20ins (n=20)	P value	
Positive rate of EGFRex20ins (%)	2.6	4.6			1.1	2.3			<0.01*
Gender				0.93*				>0.99*	0.45*
Male	1,636 (45.4)	724 (44.3)	33 (2.0)		780 (43.7)	382 (49.0)	9 (1.2)		
Female	1,970 (54.6)	1,353 (68.7)	62 (3.1)		1,005 (56.3)	482 (48.0)	11 (1.1)		
Age (years)	74 [22–86]	61 [22–86]	57 [22–79]	<0.0001*	63 [22–92]	63 [29–92]	55 [44–80]	0.36*	0.09*
≤50	618 (17.1)	330 (53.4)	44 (7.1)		269 (15.1)	144 (53.5)	5 (1.9)		
>50	2,988 (82.9)	1,747 (58.5)	51 (1.7)		1,516 (84.9)	720 (47.5)	15 (1.0)		
Smoking history				0.92*				>0.99*	0.56*
Non smoker	2,349 (65.1)	1,547 (65.9)	68 (2.9)		911 (51.0)	574 (63.0)	14 (1.5)		
Smoker	1,058 (29.4)	428 (40.5)	20 (1.9)		645 (36.1)	244 (37.8)	6 (0.9)		
NA	199 (5.5)	102 (51.3)	7 (3.5)		229 (12.9)	46 (20.1)	0		
Pathological types				0.73§				0.66§	0.64§
AdC	3,102 (86.0)	2,009 (64.8)	94 (3.0)		1,586 (88.9)	850 (53.6)	20 (1.3)		
SCC	57 (1.6)	11 (19.3)	0		100 (5.6)	8 (8.0)	0		
ASC	17 (0.5)	10 (58.8)	1 (5.9)		2 (0.1)	0	0		
NSCLC	430 (11.9)	47 (10.9)	0		97 (5.4)	6 (6.2)	0		
Clinical stage				0.05§				0.31§	<0.0001§
I	2,334 (64.7)	1,505 (64.5)	76 (3.3)		461 (25.8)	278 (60.3)	4 (0.9)		
II	226 (6.3)	102 (45.1)	6 (2.7)		62 (3.5)	22 (35.5)	1 (1.6)		
III	423 (11.8)	247 (58.4)	6 (1.4)		392 (22.0)	161 (41.1)	3 (0.8)		
IV	124 (3.4)	66 (53.2)	1 (0.8)		760 (42.6)	359 (47.2)	10 (1.3)		
NA	499 (13.8)	157 (31.5)	6 (1.2)		110 (6.1)	44 (40.0)	2 (1.8)		

Data are presented as median [range] or n (%). P value[a]: NGS EGFR Ex20ins *vs.* ARMS EGFR Ex20ins; *, Fisher's exact test; §, Chi-square test for trend. NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor; NGS, next generation sequencing; ARMS, amplification refractory mutation system; Ex20ins: exon 20 insertion; NA, not available; AdC, adenocarcinoma; ACC, squamous carcinoma; ASC, adenosquamous carcinoma.

34 unique *ex20ins* variants were identified by NGS (*Table 1*). The most frequent variant of *EGFR ex20ins* was *A767_V769dup* (29.5%, 28/95), followed by *S768_D770dup* (15.8%, 15/95), *N771_H773dup* (7.4%, 7/95) and *P772_H773dup* (7.4%, 7/95). These four types of mutations each accounted for over 5% of all *EGFR ex20ins* mutations. Additionally, 25 types of *EGFR ex20ins* variants were present in only one patient each, and interestingly, eight of which has never been reported according to Catalogue of Somatic Mutations in Cancer (https://cancer.sanger.ac.uk/cosmic) and cBioPortal databases (http://www.cbioportal.org/). *EGFR ex20ins* variants can be divided into two types: near-loop (*A767-P772*) and far-loop (*H773-C775*), based on the site of *EGFR ex20ins* helical region. Twenty-six kinds of *EGFR ex20ins* near-loop variants were found in 83 patients, while only eight kinds of *EGFR ex20ins* far-loop variants were found in 12 patients. *EGFR ex20ins* mutations detected by NGS are summarized in *Figure 2* and *Table 1*.

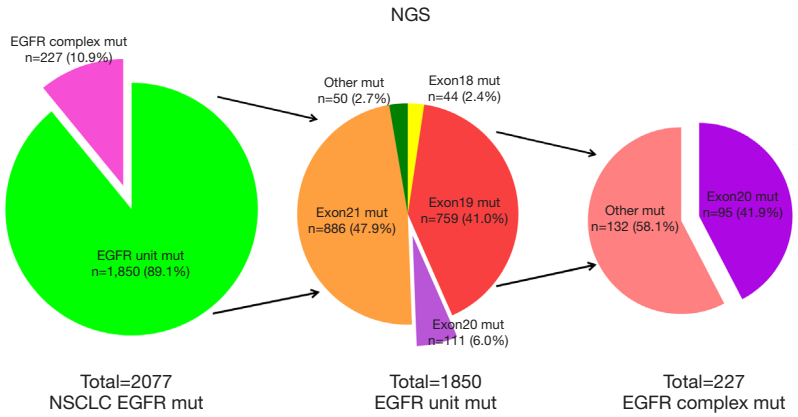

Figure 1 Frequency and distribution of 2077 EGFR mutations in 3,606 NSCLC detected by NGS. NGS, next generation sequencing; EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer.

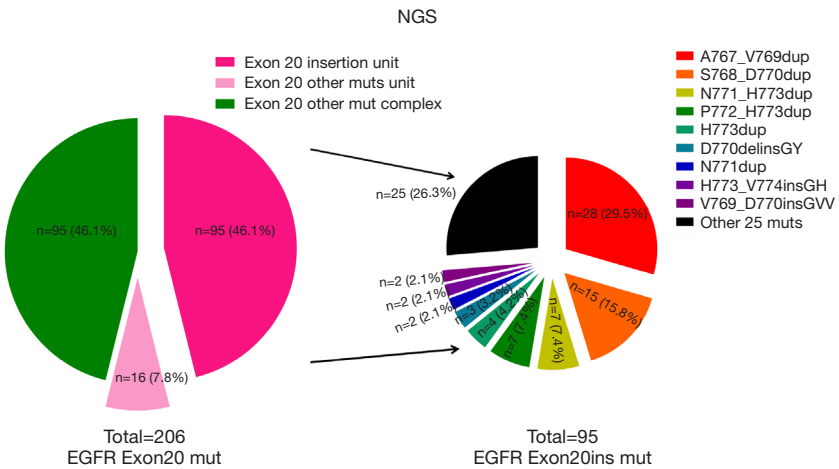

Figure 2 Frequency of EGFR exon 20 insertions detected by NGS. NGS, next generation sequencing; EGFR, epidermal growth factor receptor.

EGFR mutations and ex20ins in NSCLC patients detected by ARMS

A total of 864 *EGFR* mutations, including 825 unique and 39 complex variants, were identified by ARMS, accounting for 48.4% of the 1,785 NSCLC patients. In 825 *EGFR* unique mutations, *exon 18* mutation (n=23, 2.8%), *exon 19* mutation (n=358, 43.4%), *exon 20* mutation (n=27, 3.3%) and *exon 21* mutation (n=417, 50.5%) were demonstrated. In 39 *EGFR* complex mutations, *exon 20* accounted for 87.2% (34/39) (*Figure 3*). Similar with NGS group, most patients were *T790M* combined with *L858R* or *ex19del*.

Among the 61 *EGFR exon 20* mutation patients, 19 unique *EGFR ex20ins* mutations, eight unique *exon20* other mutations and one complex *EGFR ex20ins* mutations co-occurred with *EGFR exon21* mutation, 33 complex *exon20* other mutations were identified (*Figure 3*). In total, 20 patients with *EGFR ex20ins* were identified by ARMS, contributing 1.1% of all NSCLC cases (20/1,785) and 2.3% of *EGFR*-mutant cases (20/864), respectively (*Table 2*).

The comparison of frequencies of EGFR ex20ins in NSCLC patients detected by NGS and ARMS-PCR

Base on the above results, it is obvious that the positive rate of *EGFR ex20ins* mutations detected by ARMS method was significantly lower than that by NGS method, and the differences were statistically significant (P<0.01). In order to explore the potential causes, we carefully checked the

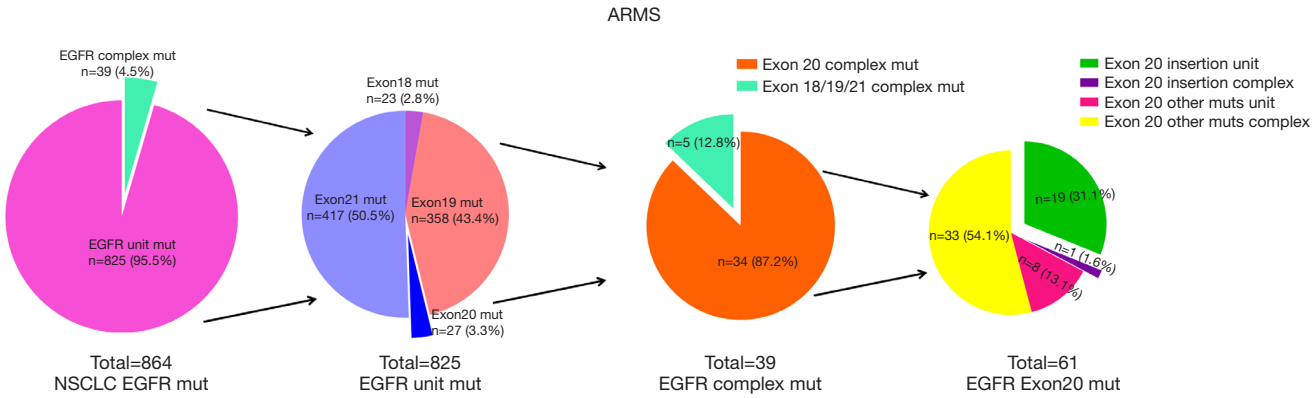

Figure 3 Frequency and distribution of 864 EGFR mutations and 61 EGFR exon 20 mutation in 1,785 NSCLC detected by ARMS. ARMS, amplification refractory mutation system; EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer.

design principle of ARMS method and found that it can just detect only 18 known *EGFR ex20ins* variants as shown in *Table 1*. Compared with 34 unique *EGFR ex20ins* variants in our study by NGS, only seven variants could be detected by ADx-ARMS *EGFR* kit mutation used in this study, accounting for 42.1% (40/95) of all *EGFR ex20ins* mutations by NGS. It meant that if ARMS was used to detect the 3,606 Chinese NSCLC patients tested by NGS, the false negative rate of *EGFR ex20ins* mutations reached 57.9%.

In addition, the ability to detect *EGFR ex20ins* mutations in two FDA-approved PCR kits, Therascreen and Cobas *EGFR* v2, was also evaluated. As shown in *Table 1*, Therascreen can detect three variants of *ex20ins*, and Cobas *EGFR* v2 can detect five types of *ex20ins*. Unfortunately, only one and two out of our 34 unique *EGFR ex20ins* variants could be detected, accounting for 1.1% (1/95) and 30.5% (29/95) of all *EGFR ex20ins* mutations based on our NGS data, respectively. It meant that if these two tests were used in Chinese NSCLC patients, the false negative rate of *EGFR ex20ins* mutations could be ranged about 69.5% to 98.9%.

Discussion

In NSCLC patients, the *EGFR* oncogenic driver mutations occur mainly in *exons 18*, *19*, *20* and *21*, which encode the tyrosine kinase domain. The in-frame insertion within *exon 20* is the third most frequent class of *EGFR* mutations, accounting for 4–12% of *EGFR* mutations (14,28,29). Patients with *EGFR ex20ins* fundamentally have a shorter survival time compared with those who have common *EGFR* mutations due to the general lack of sensitivity to *EGFR* TKIs (11). Novel *EGFR* TKIs targeting *EGFR ex20ins* are emerging. Recently, amivantamab (JNJ-372) have been approved by the US FDA for the treatment of advanced or metastatic NSCLC patients with *EGFR ex20ins* mutations (30). Mobocertinib (TAK-788) has been granted priority review by the FDA for the same indication as amivantamab. In a phase 1/2 study, mobocertinib demonstrated antitumor activity in patients with NSCLC with diverse *EGFR ex20ins* variants with a safety profile consistent with other *EGFR* inhibitors in the first two parts of a three-part phase I/II study (ClinicalTrials. ID: NCT02716116) (31). In another study, mobocertinib also showed antitumor activity in patients with *EGFR ex20ins*-positive NSCLC (32,33). Although the sensitivity of most *EGFR ex20ins* to *EGFR* TKIs was generally lower, *EGFR-A763_Y764ins FQEA* is a unique *EGFR ex20ins* mutation which is sensitizing to clinically available *EGFR* TKIs (34). Thus, *EGFR ex20ins* are shown to have different sensitivity to different *EGFR* TKIs. Accurate detection of *EGFR ex20ins* is important for the treatment of NSCLC patients. Therefore, the latest NCCN guidelines recommend explicitly reporting specific variant types of *EGFR ex20ins* in the test reports, better guiding the choice of treatment options (27).

Previous studies showed that the frequency of *EGFR ex20ins* mutations in Chinese NSCLC patients is about 2.21–2.27% (25,35,36). In this study, a total of 95 *EGFR ex20ins* mutations were identified in a subset of 3,606 NSCLC patients detected by NGS and 20 *EGFR ex20ins* were in 1,785 NSCLC patients by ARMS, with the frequency of *EGFR ex20ins* in Chinese NSCLC patients as 2.6% and 1.1%, respectively. The frequency of *EGFR ex20ins* of NGS detection in our results was slightly higher than that in previous studies and significantly higher than

that by ARMS. The result was consistent with He *et al.* (37). They found the mutation rate of ex20ins by ARMS-PCR detection (1.3%) were statistically significantly lower than that of NGS detection (2.0%). In NGS patients, 46.3% (44/95) *EGFR ex20ins* patients were ≤50 years old and most of them were in clinical stages I and II. However, in ARMS patients, only 25.0% (5/20) *EGFR ex20ins* patients were ≤50 years old and most of them were in clinical stage III or IV.

The types of *EGFR ex20ins* mutations are numerous. Targeting *EGFR ex20ins* will be more complicated than targeting the classic *EGFR* mutations, *L858R* and *ex19del*, as the insertions are too diversified to support a one for-all solution. In the previous studies, 39–85 different molecular variants of *EGFR ex20ins* were identified in Chinese NSCLC patients with *A767_V769dup* and *S768_D770dup* being the most prevalent ones (38,39). Our study showed that 34 different subtypes of insertion variants were recorded in NGS, and the two most common *EGFR ex20ins* variants were consistent with previous studies, *A767_V769dup* (29.5%) and *S768_D770dup* (15.8%), comprised 45.3% of all *EGFR ex20ins* and can be the main targets in future drug development efforts. However, eight kinds of *EGFR ex20ins* variants were identified in our study, which were reported for the first time according to COSMIC and cBioPortal databases. These results suggested that accurate detection of *EGFR ex20ins* and subsequent individualized treatment are still full of challenges.

NGS has the advantages of high flux and diverse detection types in detecting gene mutations. However, it also meets the problem of high cost in time and economy. From nuclein extraction to report diagnosis, it will take up about 14 days and the high expense of machine and reagent make it hard to popularize in the primary hospitals in China. Although ESMO 2022 suggested that NGS assay should be prioritized for *EGFR ex20ins* analysis to allow broader detection and characterization, ARMS is still one of the most common methods to detect *EGFR* gene mutations in Chinese NSCLC patients because of its simple operation, faster reports, reliable and cost-effectiveness. However, it has several limitations to detect *EGFR ex20ins* mutations. First, the reason for ARMS with the high sensitivity is its peculiar primer design, but it's also the limitation. The primers make ARMS applies only in the detection of known mutations. The currently approved kits in China only cover up to 18 specific somatic insertion mutations in *exon 20* of the *EGFR* oncogene (*Table 1*), while more than 100 *EGFR ex20ins* mutation types are currently identified and more novel previously unidentified mutations are being discovered. In our study, the second most frequent variant of *EGFR ex20ins* detected by NGS, *S768_D770dup*, was not included in the ADx-ARMS-*EGFR* kit. Based on the results of the NGS, the frequency of the 18 mutations detected by the ARMS kit were not high, the primers are designed based on the Western population, it may lead to *EGFR* mutations missed in the Chinese population. Second, *EGFR ex20ins* were shown to have different sensitivity to different *EGFR* TKIs. For example, one study found that poziotinib sensitivity was highly dependent on the insertion location, with near-loop insertions (amino acids A767 to P772) being more sensitive than far-loop insertions, an observation confirmed clinically with objective response rates (ORRs) of 46% and 0% observed in near versus far-loop, respectively (40). In this study, we found eight kinds of *EGFR ex20ins* far-loop variants and 26 kinds of near-loop variants by NGS. Thus, a structure-function-based approach may improve the prediction of drug sensitivity to targeted therapies for *EGFR ex20ins* mutations (41). The NCCN guidelines recommend reporting specific variant types of *EGFR ex20ins* in test reports (42). However, the ARMS testing could not report the specific variant type. Third, the ARMS detection kit currently does not cover the newly discovered rare lung cancer targets, such as *FGFR2* fusion, *NGR1* fusion, etc. Considering the advantages and disadvantages of NGS and ARMS, National Health Service (NHS) England guidance that describes a salvage testing pathway for patients with advanced lung cancer who would not survive to see the potential beneficial repercussions of NGS-based mutation detection. It allows local testing to continue by rapid PCR methods in a context of genomic testing in centralized laboratory hubs (43). Therefore, the commercial allele-specific ARMS testing solutions cannot fully meet clinical needs for *EGFR ex20ins* detection, and need iterative upgrading.

Conclusions

More and more variants of *EGFR ex20ins* mutations have been identified, and NGS has obvious advantages and is strongly recommended for the detection of *EGFR ex20ins* mutations. Using the existing PCR-based detection kit to detect *EGFR ex20ins* mutations has a high false negative rate. Considering the fast and cost-effective ARMS detection method, it suggests that the primers design should be updated according to the characteristics of *EGFR ex20ins* mutations in Chinese NSCLC patients.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Ethical approval was obtained from the Research Ethics Committee of Fudan University Shanghai Cancer Center (No. 050432-4-2108*). Informed consent was waived due to the retrospective nature of the study. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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