Original Article

Exploratory analysis of introducing next-generation sequencing-based method to treatment-naive lung cancer patients

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Background: The utilization of cancer-linked genetic alterations for categorizing patients against optimal treatment is becoming increasingly popular, especially in non-small cell lung cancer (NSCLC). However, disadvantages of the conventional techniques, such as the low throughput and limited detectable alteration types, lead to the demand of large-scale parallel sequencing for different forms of genetic variants.

Methods: We evaluated the potential of performing next-generation sequencing (NGS)-based methods in a cohort of 61 treatment-naive NSCLC patients to profile their driver mutations, using a panel consisting of 8 well-established driver genes of lung cancer.

Results: Our data revealed that 80% of patients harbored driver mutations. Moreover, our data revealed a few rare mutations, such as *BRAF* K601E and *EGFR* exon 20 insertion, which cannot be detected using commercially available single gene testing kits of conventional methods. We detected one patient with dual driver mutations. Next, correlations between driver mutations and clinical characteristics were interrogated in this cohort. Our results revealed that *EGFR* alterations were positively correlated with early stage, adenocarcinoma, alveolar and papillary component, TTF1 expression, and negatively correlated with P40 and Ki67 expression. *ERBB2* alterations were associated with younger age and micro-invasive feature of tumor. Rearrangements of *ALK* indicated tumor relapse.

Conclusions: Our study highlights the potential of NGS-based methods in treatment-naive patients, thus paving its way for routine clinical use. Investigation of clinical correlation of driver mutations might be helpful for clinicians in cancer diagnosis and has implications for seeking patients with specific gene alteration in clinical studies.

Keywords: Non-small cell lung cancer (NSCLC); treatment-naive; next-generation sequencing (NGS); targeted therapy; driver mutations

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Introduction

The treatment of cancer has revolutionized in the past decade with the development of therapies directed at specific genetic alterations, serving as a panel of druggable targets. Among all cancer types, non-small cell lung cancer (NSCLC), harboring a spectrum of mutations, has the richest targeted therapy options, ranging from compounds in clinical development to FDA-approved drugs, targeting multiple oncogenic driver genes, including epidermal growth factor receptor (*EGFR*) (1,2), anaplastic lymphoma kinase (*ALK*) (3), c-ros oncogene 1 (*ROS1*), *ERBB2*, *BRAF*, *MET* and *RET* (4). The characterization of NSCLC into subtypes based on their genetic alterations has significantly improved the therapeutic efficacy of targeted therapies and disease outcomes in a subgroup of patients (1,2). Accurate companion diagnosis is necessary for patient selection prior to commencing these treatment options (5-7).

Currently, single gene testing methodologies, including but not limited to ARMS-PCR, FISH and IHC are often utilized in treatment-naive patients to profile driver mutations, such as *EGFR* mutations and *ALK* fusions for classifying patients against optimal treatments (8-10). However, each of conventional technique is associated with its own disadvantages, including the low-throughput nature, limitations in detecting certain types of aberrations and the need for subjective judgments during data analysis. DNA next-generation sequencing (NGS) allows for large-scale parallel sequencing and has proved to be an effective and accurate tool for the parallel profiling of different forms of genetic abnormalities including mutations, fusions, and amplifications across a large number of genes (11). It also allows for the identification of novel mutations which cannot be identified using PCR-based methods. Countless studies have reported its utility in diagnosis and treatment guidance across a wide spectrum of cancer types. Currently in lung cancer, NGS-based methods are often performed on samples obtained from patients who have progressed on prior treatments due to the likelihood of harboring multiple mutations.

In this study, we performed capture-based ultra-deep sequencing on 61 surgically-resected samples obtained from treatment-naive patients using a panel consisting of 8 well-established driver genes for lung cancer to investigated driver mutations associated with each patient. This study evaluated the potential of utilizing NGS-based methods in treatment-naive patients and paved its way in routine clinical use in treatment-naive patients. Based on driver mutations identified using NGS, we interrogated the correlations between driver mutations and clinical characteristics in this Chinese treatment-naive cohort.

Methods

Patient selection

Between January 2016 and September 2016, 61 treatment-naive patients with resectable NSCLC were enrolled in this study. This study was approved by the Institutional Review Board at Zhangjiagang First People's Hospital. All patients gave informed consent to participate in the study and gave permission for the use of tumor tissues.

NGS library preparation and sequencing

DNA was extracted using QIAamp DNA FFPE tissue kit (Qiagen) according to manufacturer's instructions. DNA concentration was measured using Qubit dsDNA assay. DNA shearing was performed using Covaris M220, followed by end repair, phosphorylation and adaptor ligation. Fragments of size 200–400 bp were selected by bead (Agencourt AMPure XP Kit, Beckman Coulter, California, USA) followed by hybridization with capture probes baits, hybrid selection with magnetic beads and PCR amplification. A bioanalyzer high-sensitivity DNA assay was then performed to assess the quality and size of the fragments and indexed samples were sequenced on Nextseq500 sequencer (Illumina, Inc., California, USA) with pair-end reads.

Genetic profiles of all tissue samples were assessed by performing capture-based targeted deep sequencing using the 8-gene panel, covering 76 kb of human genomic regions. The 8-gene panel can detect oncogenic driver mutations of *EGFR*, *ALK*, *BRAF*, *MET*, *RET*, *ROS1*, *ERBB2* and *KRAS*.

Sequence data analysis

Sequence data were trimmed with Trimmomatic (12) for adaptor and mapped to the human genome (hg19) using BWA aligner 0.7.10 (13). Local alignment optimization, variant calling and annotation were performed using Genome Analysis ToolKit (GATK) 3.2 (14), Picards (http://picard.sourceforge.net/) and VarScan (15). Variants were filtered using the VarScan fpfilter pipeline. At least 5 supporting reads were needed for INDELs,

Table 1 Summary of baseline patient characteristics

Patient characteristics	Number (%)
Total	61
Gender	
Female	30 (49.2)
Male	26 (42.6)
Unknown	5 (8.2)
Age (year)	
Median	61.5
Range	25–84
Smoking history	
Yes	13 (21.3)
No	43 (70.5)
Unknown	5 (8.2)
Histological types	
LUAD	48 (78.7)
LUSC	8 (13.1)
Unknown	5 (8.2)
TNM stage	
Ia	39 (63.9)
Ib	5 (8.2)
II	1 (1.6)
IIIa	1 (1.6)
IIIb	2 (3.3)
IV	3 (4.9)
Unknown	10 (16.4)

LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma.

while 8 supporting reads were needed for SNVs to be called. According to the ExAC, 1000 Genomes, dbSNP, ESP6500SI-V2 database, variants with population frequency over 0.1% were grouped as SNP and excluded from further analysis. Remaining variants were annotated with ANNOVAR and SnpEff v3.6. DNA translocation analysis was performed using both Tophat2 and Factera (16) and CNVs were analyzed with inhouse algorithm based on sequencing depth.

Statistical analysis

The statistical analyses were performed using Software R. Statistical differences were calculated and presented using paired, two-tailed Student's t-test in P value. For all statistical tests, $P<0.05$ was considered statistically significant.

Results

Patient characteristics

From January 2016 to September 2016, 61 treatment-naive patients with resectable NSCLC were enrolled in this study. The median age was 61.5 years, ranging from 25–84 years old. Thirty patients were female and 26 were male. Thirteen patients had a history of smoking and 43 patients were nonsmokers. Forty-eight cases were diagnosed as adenocarcinoma; 8 were squamous cell carcinoma (*Table 1*). Thirty-nine patients were classified as stage IA, accounting for 63.9% of the cohort; 5 patients were diagnosed (8.2%) at stage IB, 1 at stage II, 1 at stage IIIa, 2 at stage IIIb, and 3 patients with stage IV.

Mutation spectrum

To elucidate driver mutations associated with each patient, we performed capture-based ultra-deep targeted sequencing using a panel consisting of all exons of 8 well-established driver genes for lung cancer, spanning 76 kb of human genome. We identified mutations from 80% (49/61) patients and the remaining 12 (20%) patients had no mutation identified from this panel. Collectively, 79 genetic variants were identified including 43 single nucleotide variations (SNVs), 28 insertions or deletions (INDELs), 4 copy-number amplifications (CNAs), and 4 translocations.

EGFR is the most frequently mutated gene, accounting for 44% of all variants identified (*Figure 1A*). Four types of *EGFR* mutations were detected in this study, including in-frame deletions in exon 19, single missense substitution mutation L858R, G719A, and exon 20 insertions. Of the *EGFR* somatic mutations, 83% of them were TKI sensitive mutations, including exon 19 deletions, L858R and G719A; 14% were EGFR-TKI resistant mutations, exon 20 insertion. Moreover, one patient was detected with *EGFR* G719A mutation (*Figure 1B*).

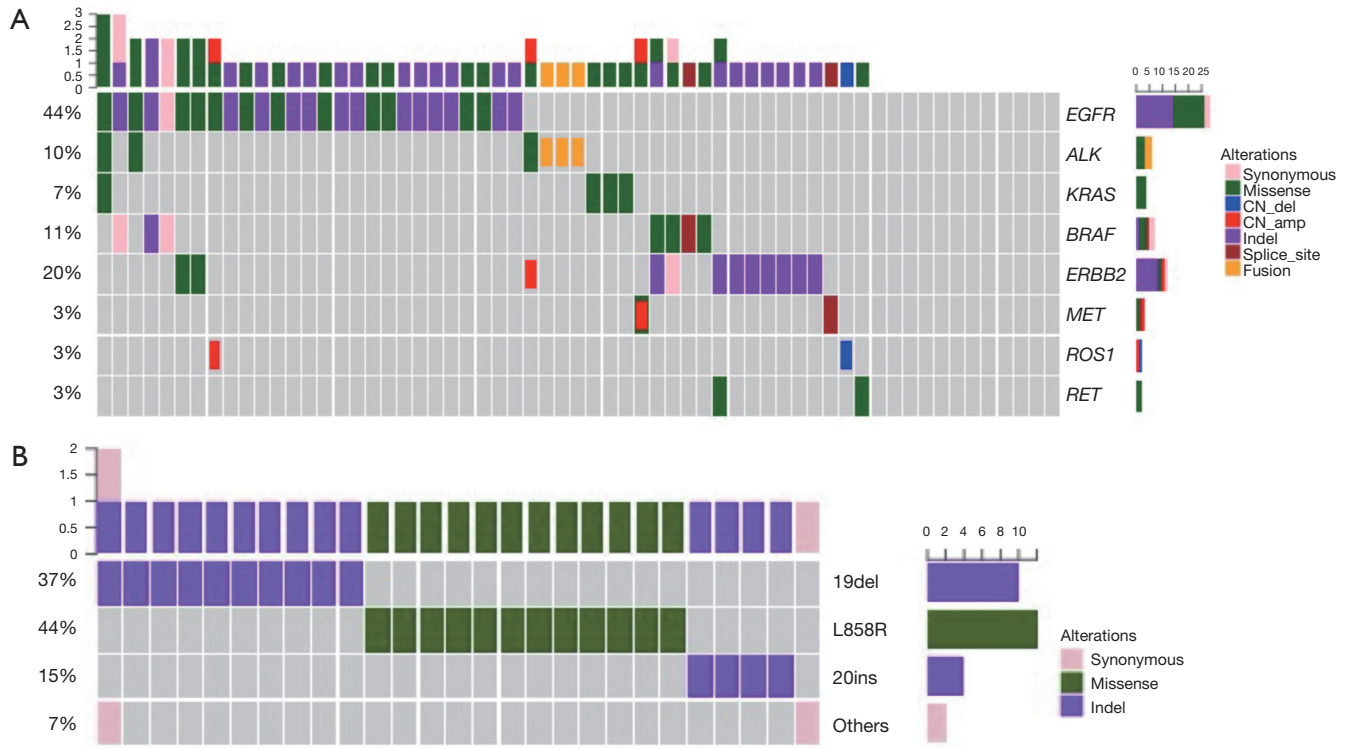

Figure 1 Mutation spectrum. (A) Driver mutations detected from each patient were plotted. Different colors denote different forms of mutations. Bars on the right side of the mutation spectrum summarize the number of patients harboring certain mutations; top bars summarize the number of mutations a patient carries. (B) Detailed presentation of *EGFR* mutations.

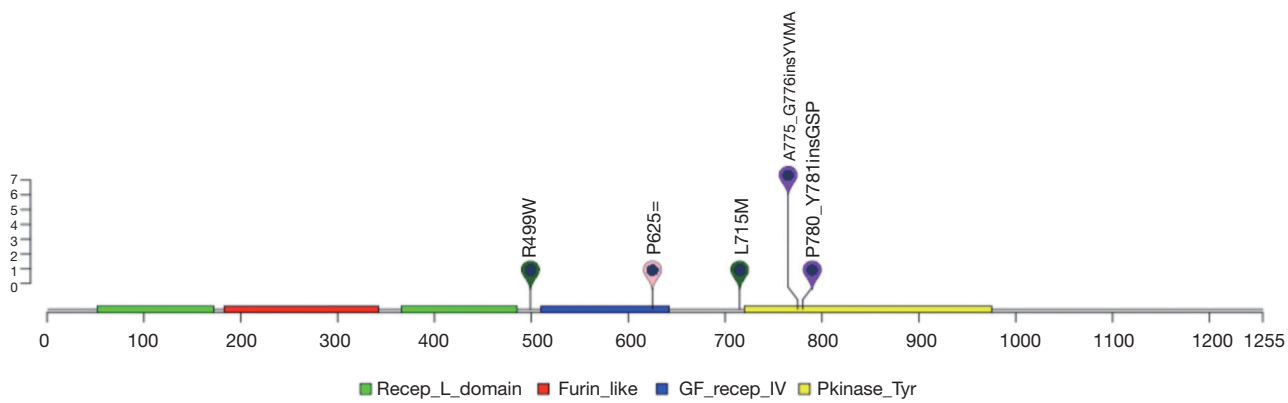

Figure 2 Schematic diagram for *ERBB2*. Different domain of *ERBB2* was depicted. Mutations all located in ERBB2 tyrosine kinase domain.

Twelve patients (20%) were identified with *ERBB2* mutations. Among them, 13% of patients (8/61) harbored *ERBB2* exon 20 insertion, higher than reported by previous studies, ranging from 2% to 4% (17-19). This variant located in ERBB2 tyrosine kinase domain and can affect protein function in a large extent (*Figure 2*).

One patient was detected with *MET* skipping splicing and another with *MET* copy number amplification. *MET* exon 14 skipping promotes *MET* RNA-splicing-based skipping, thus leading to MET kinase activity stimulation.

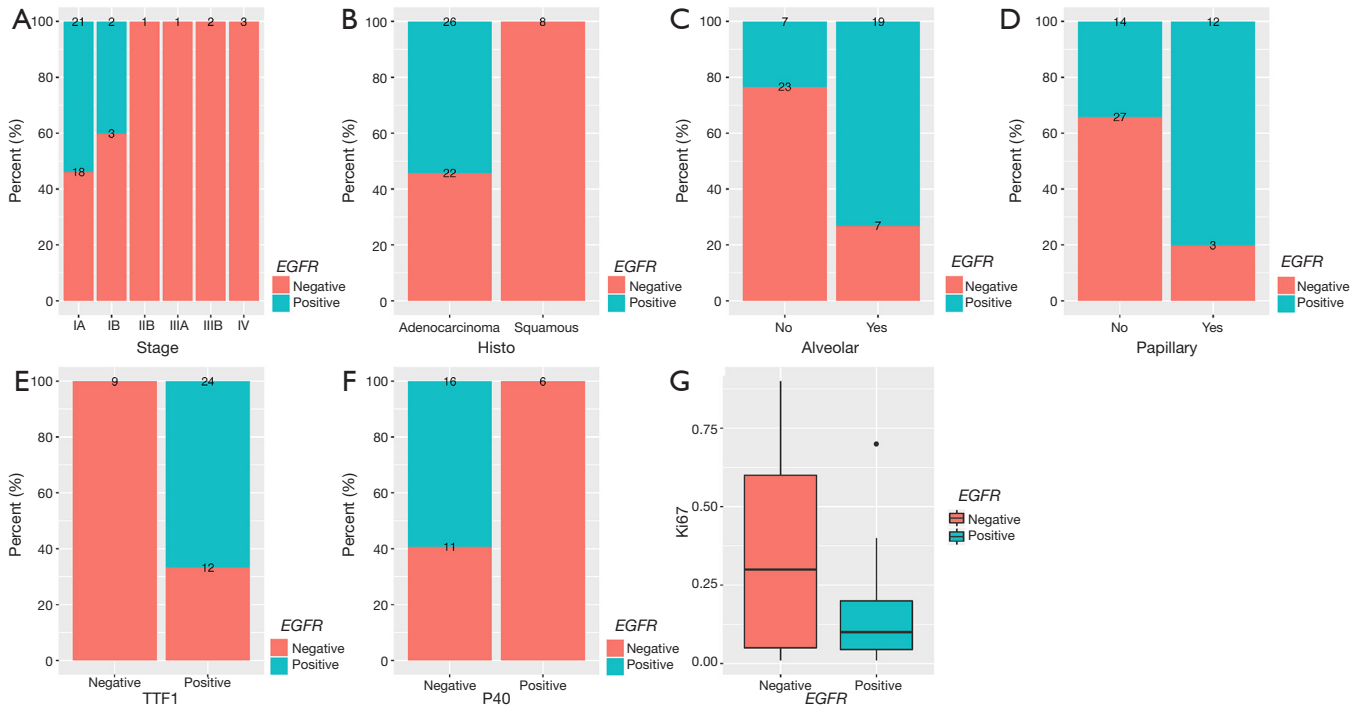

Figure 3 Correlation between *EGFR* alterations and clinical features. (A) *EGFR* mutation was correlated with early-stage. (B) *EGFR* alterations were more prone to occur in adenocarcinoma than squamous cell carcinoma. (C,D) Tumors with *EGFR* mutation were more likely to have alveolar (C) and papillary (D) components. (E,F,G) Tumors with *EGFR* mutations commonly have overexpressed TTF-1 (E) and lower expressed P40 (F) and Ki67 (G).

Exon 14 encoded protein can recruit ubiquitin ligase CBL for a result of MET degradation (20-22). *MET* exon 14 deficiency will cause MET protein stability and persistent activation of downstream signaling (23). Taken together, exon 14 splicing alteration gives rise to oncogenic MET activation for tumorigenesis.

Seven patients carried *BRAF* mutations; among them, one patient harbored a rare *BRAF* mutation K601E, which is not included in commercially available *BRAF* mutations detection kit. *BRAF* K601E, located between two major BRAF phosphorylation sites T599 and S602 in the activation loop in the vicinity of the classic mutation V600E, has been reported in melanoma (24) and follicular thyroid carcinoma (25).

ALK fusion was found in three samples (5%), and *KRAS* single missense mutation G12D in four patients (6.6%). *ROS1* copy number deletion and *RET* mutation were also detected in our cohort. In addition, 12 patients (12/61) had no mutation identified from this panel.

Of all the patients harbored driver mutation, only one patient had dual driver mutations, harboring both *EGFR* L858R and *KRAS* G12D mutation (*Figure 1A*), in an agreement with previous findings that driver mutations exhibit mutually exclusive correlation in treatment-naive patients (26).

Correlation between driver mutations and clinical characteristics

Next, we investigated the correlation between driver mutations and clinical parameters (including but not limited to age, gender, smoking history, histology) and expression of immunohistochemistry (IHC) markers (including but not limited to CK7, TTF-1, p40, CD56) in this Chinese treatment-naive lung cancer cohort.

First, we interrogated the correlation of *EGFR* mutations and clinical features. We revealed that high prevalence of *EGFR* mutations was more commonly found in patients diagnosed at early-stage (P=0.0093, *Figure 3A*). *EGFR* mutation was significantly correlated with adenocarcinoma histology than squamous cell carcinoma (P=0.0052, *Figure 3B*). Tumors with *EGFR*-positive mutation were more likely to

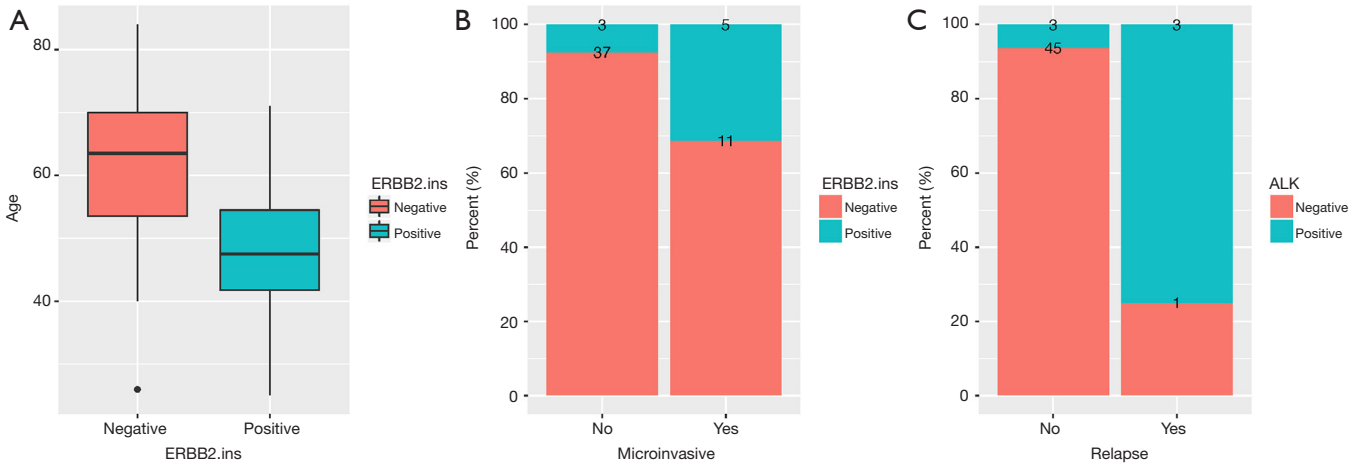

Figure 4 Correlation of *ERBB2* or *ALK* alterations and clinical features. (A) *ERBB2* were more prone to occur in younger age. (B) Tumors with *ERBB2* mutations were associated with micro-invasive potential. (C) *ALK*-mutated tumors have more probability to relapse.

have alveolar (P=0.0004, *Figure 3C*) and papillary features (P=0.0053, *Figure 3D*) than that of *EGFR*-negative. As to expression of IHC markers, we observed that patients harboring *EGFR* mutation commonly had high expression of TTF1 (P=0.0003, *Figure 3E*), and low level of P40 (P=0.0184, *Figure 3F*) and Ki67 (P=0.0109, *Figure 3G*), compared to *EGFR*-negative patients.

Clinical relevance of *ERBB2* and *ALK* genomic alterations were also investigated. The alteration of *ERBB2* was positively correlated with younger age (P=0.0077, *Figure 4A*). Tumors detected with positive-*ERBB2* were significantly correlated with micro-invasive features than *ERBB2*-negative tumors (P=0.0351, *Figure 4B*). For patients harboring *ALK* alterations, they were more prone to relapse than patients without *ALK* alterations (P=0.0035, *Figure 4C*).

Discussion

In this study, we investigated the potential of introducing NGS-based testing methods to treatment-naive patients in a cohort of 61 treatment-naive patients by performing capture-based ultra-deep targeted sequencing using a panel consisting of 8 well-established driver genes for lung cancer. Our data revealed that 80% of patients carried driver mutation, which exhibits a mutually exclusive relationship with each other except for one case. Our data also reveal rare mutations, which can not be identified using PCR-based methods. This study highlights the potential of NGS in treatment-naive NSCLC patients. Moreover, most of previous studies were focusing on distinct molecular feature of lung cancers. Here, we clarified the spectrum of well-established oncogenic driver mutations in a Chinese treatment-naive lung cancer cohort, and analyzed the correlation between driver alterations and clinical characteristics.

Currently, single gene testing is often performed on treatment-naive patients to detect mutations to guide subsequent treatment. NGS-based methods are often used in patients who have developed resistance to prior therapies. Single gene testing methods, such as amplification refractory mutation system polymerase chain reaction (ARMS-PCR), fluorescent in situ hybridization (FISH) and IHC, are associated with a few pitfalls, including but not limited to detection of known variants, limited ability in multiplexing, and involving subjective judgement (27). Our study demonstrated that NGS-based methods allow for large-scale parallel sequencing to detect a wide range of genetic mutations simultaneously.

Using NGS, we identified numerous mutations that are not covered by commercially available testing kits, such as *EGFR* exon 20 insertions, *ERBB2* exon 20 insertions, *MET* exon 14 skipping and *BRAF* K601E. Although the traditional method ARMS-PCR is often utilized to detect mutations in treatment-naive patients, all above mentioned mutations are not covered by commercially available kits. Thus, patients harboring such mutations may miss therapeutic opportunities if subject to test using commercially available single gene testing kits. It has been

reported that patients with *MET* exon 14 skipping are responsive to crizotinib and positive detection of exon 14 could guide following targeted therapy for clinical benefit achievement (28). *BRAF* K601E located between two phosphorylation sites in BRAF activation domain (29,30). Preclinical studies reported that MEK inhibitor, trametinib (Mekinist, GSK1120212) can block the downstream signaling induced by *BRAF* K601E mutation (31,32), which implies that *BRAF* K601E mutation maybe sensitive to trametinib. Therefore, NGS should be recommended when multiple genes need to be tested.

In addition, our data revealed that 20% of patients in this cohort did not carry mutation from this panel. Several potential reasons might be responsible for this. This may attribute to tumor heterogeneity that the sampled tissue did not carry driver mutations. Another possible explanation can be the relatively low tumor content in the biopsy tissue. Furthermore, there are a significant percentage of patients who do not harbor driver mutation. Their cancer is not driven by driver mutations but another mechanisms or mutations which are not covered by our panel. Collectively, in this study, we revealed the essentialness of introducing NGS-based testing methods to treatment-naive patients.

Although NGS has been widely regarded as a powerful tool to accurately detect gene alterations in a high-throughput scale, it still has its own limitations (33,34). First, the period for NGS procedure is commonly longer for clinical diagnosis when compared to conventional methods such as ARMS-PCR and FISH. Second, the cost is an obstacle for patients to choose NGS for mutation identification. Third, the accuracy of new platform may less accurate than conventional method on specific mutation type identification. However, although challenges were created by these limitations, we should keep in mind that NGS will continue to be improved and optimized with respect to these disadvantages.

Nowadays, translational medicine has progressed to the point where patients can be stratified basing on the correlation of clinically relevance and molecular features (35,36). This kind of association might be helpful for diagnosis and patient selection for specific tumor mutations in clinical trials. There have been many studies examining the relationship between patient characteristics and oncogenic driver mutations in NSCLCs. However, several data were conflicting among different studies (37-40). Here, on the basis of oncogenic driver mutation identified by NGS, we conducted this kind of investigation in our study and tried to interrogate the underlying clinical features of tumors with different driver mutations in this treatment-naive Chinese NSCLC cohort. The presence of *EGFR* mutations in a subset of NSCLC were associated with a favorable prognosis in cancer patients treated with EGFR tyrosine kinase inhibitors (TKIs) (1,41). Identification of *EGFR* mutated-patients may indicate potential sensitive patients who would be benefit from EGFR-TKI. In this study, we found that *EGFR* mutations were correlated with early stage, indicating that this well-known oncogene driver mutation was acquired in early step of pulmonary carcinogenesis. Next, we evaluated whether morphological features reflect *EGFR* mutation status. We found that *EGFR* mutations were more frequently observed in tumor with alveolar or papillary components than that without such components. Our results revealed that tumors with overexpressed TTF-1 and low expressed P40 and Ki67 would have more *EGFR* mutations, which indicated that these immunohistological markers such as TTF-1, P40 and Ki67 can predict *EGFR* mutation. These results suggested that *EGFR* mutation test should be performed in tumors with such clinical features. Gene alterations in the *ERBB2* or *ALK* also identify distinct subsets of NSCLC and represent a therapeutic target with already proven sensitivity to *ERBB2* or *ALK* inhibitors in clinical settings. Here, we found *ERBB2* mutations were correlated with younger age and micro-invasive potential. *ALK* mutations indicated further relapse probability of tumors. Although discrepancies could be introduced by the limited number of cases, these findings suggested that these clinical characteristics could be served as indicators for presence of *ERBB2* and *ALK* mutations.

In conclusion, treatment-naive patients often undergo a few single gene tests to identify driver mutations. Several well-established driver mutations cannot be detected using commercially available single gene testing kit. We demonstrated a preferable way to profile a wide range of genetic alterations in tissue biopsy of treatment-naive NSCLC patients by NGS, highlight the needs for treatment-naive patients to undergo genomic profiling. In addition, the investigation of correlation between oncogenic driver mutations and clinical characteristics would implicate mutations examination in specific patients, which further guides more precise targeted therapy and helps patients to achieve better clinical benefit.

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Footnote

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

Ethical Statement: This study was approved by the Institutional Review Board at Zhangjiagang First People's Hospital (No. 2017003). Written informed consent was obtained from patients for this study and publication of this manuscript.

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