OncoTargets and Therapy

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ORIGINAL RESEARCH

Comparison of Next-Generation Sequencing and Ventana Immunohistochemistry in Detecting ALK Rearrangements and Predicting the Efficacy of First-Line Crizotinib in Patients with Advanced Non-Small Cell Lung Cancer

This article was published in the following Dove Press journal:
OncoTargets and Therapy

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Introduction: Reliable diagnostic approaches to detect ALK rearrangement are critical for selecting patients eligible for crizotinib therapy. This study aimed to compare next-generation sequencing (NGS) and Ventana immunohistochemistry (IHC) in evaluating ALK rearrangements and evaluate their impact on first-line crizotinib efficacy.

Patients and Methods: A total of 472 NSCLC patients were identified as ALK-positive by NGS and/or IHC between March 2014 and February 2020. The concordance of ALK detection, overall response rate (ORR), and progression-free survival (PFS) were analyzed for 319 patients who received front-line crizotinib.

Results: First-line crizotinib (n=319) significantly prolonged PFS in comparison with chemotherapy (n=46; 12.0 vs 6.8 months; p<0.0001). Of the 76 crizotinib-treated patients whose ALK status was assessed by both NGS and IHC, 78.9% of the patients had concordant ALK status (NGS-positive/IHC-positive), 18.4% patients were NGS-positive but IHC-negative, and 2 patients were IHC-positive but NGS-negative. Different detection assays confer no statistical difference in ORR and PFS with first-line crizotinib. The ORR in NGS only, IHC only, and both NGS and IHC was 84.3%, 90.1%, and 88.1%, respectively, while PFS was 11.4, 13.0, and 11.0 months, respectively. The ORR in NGS-positive/IHC-positive and NGS-positive/IHC-negative patients was 85.4% and 92.8%, respectively. Compared to NGS-positive/IHC-positive patients, those with NGS-positive/IHC-negative results had a trend of shorter PFS but statistical significance was not reached (mPFS, 5.9 months vs 11.5 months, p=0.43).

Conclusion: Our results demonstrate that ALK status detected by NGS and/or IHC is reliable in identifying patients with ALK-positive NSCLC who will benefit from ALK inhibitor therapy.

Keywords: ALK status evaluation, ALK IHC, ALK inhibitor

Introduction

Lung cancer is the primary cause of cancer-associated mortality worldwide, with non-small-cell lung cancer (NSCLC) accounting for approximately 85% of all lung cancer cases.[1] Anaplastic lymphoma kinase (ALK) rearrangement, a transforming fusion resulting from inversion or translocation events in chromosome 2p, is a proven molecular target and a potent oncogenic driver in approximately 5% of NSCLCs.[2,3] Based on the robust efficacy of crizotinib in previous clinical trials, the

United States Food and Drug Administration (US-FDA) had approved crizotinib as first-line treatment for patients with ALK-positive advanced NSCLC.[4–6] This highlights the need for reliable methods in assessing the ALK status to identify the subset of patients who may benefit from crizotinib therapy.[7]

Vysis ALK Break Apart fluorescence in situ hybridization (FISH) kit (Abbott Molecular, Abbott Park, IL) was approved by the US-FDA in 2011 as the gold standard for detecting ALK rearrangements.[8] However, FISH is a complex technology that requires specialized equipment and involves complicated results interpretation, which makes it an unpopular choice for routine screening of ALK rearrangement in clinical practice.[9] Over the last decade, ALK immunohistochemistry (IHC), which detects ALK protein expression, became a widely used method in pathology laboratories and gained clinical importance in selecting patients for crizotinib treatment due to its cost- and time-efficient performance. Several studies have demonstrated that ALK antibody D5F3 clone is reliable in identifying patients who benefit from crizotinib, which resulted in the US-FDA approval of Ventana ALK (D5F3) Assay in 2015 as a companion diagnostic (CDx) test with equal sensitivity and specificity to FISH.[10–13]

Recently, targeted next-generation sequencing (NGS) is becoming a clinically preferred molecular diagnostic method due to its capability to simultaneously detect multiple mutations using a small volume of specimens in a single test.[14,15] Various genomic ALK aberrations, including increased copy number, point mutations, and rearrangement, can be directly detected by NGS.[16–18] In addition to mutation status, the details of ALK fusion gene partners can also be revealed by NGS. Although previous reports have explored the utility of NGS in detecting ALK rearrangements and indicated that NGS-based ALK-positive status may predict clinical benefit with crizotinib,[19–23] the association between ALK status assessed by NGS and therapeutic response from crizotinib has not been well validated. Studies with larger sample size are needed to establish the role of NGS in selecting patients eligible for crizotinib treatment.

In this study, we analyzed a retrospective cohort with ALK-positive NSCLC who had their ALK status assessed using either NGS and/or Ventana IHC, to evaluate the predictive value of the ALK assessment using the two molecular approaches on the efficacy of first-line crizotinib therapy.

Patients and Methods

Patients

A total of 9440 patients diagnosed with NSCLC between March 2014 and February 2020 in Hunan Cancer Hospital were screened for this study. The 319 patients analyzed for clinical and survival outcomes met the following criteria: (1) pathologically confirmed NSCLC; (2) have ALK-positive tumors confirmed by either NGS (Burning Rock Biotech, Guangzhou, China) and/or IHC (Clone D5F3); and (3) received crizotinib in the first-line setting. Pathological diagnosis was performed independently by two qualified Pathologists and staging was carried out according to the staging system of the 2009 International Association for the Study of Lung Cancer (version 8). Baseline demographics and clinicopathologic information were collected for all the patients including Eastern Cooperative Oncology Group (ECOG) performance status (PS), clinical stage, and metastasis. Crizotinib was orally administered with a dose of 250 mg twice daily until the evaluation of progressive disease (PD) or unacceptable toxicity. The clinical responses were evaluated by the investigators according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.113. Progression-free survival (PFS) was measured from the first day of crizotinib administration until tumor progression or death. Approval was obtained from the ethics committee of Hunan Cancer Hospital (approval number: 2017YQ-225). Written informed consent was obtained from each patient prior to study enrollment.

Next-Generation Sequencing

NGS detection was performed as previously described.[24] Briefly, tumor DNA and circulating cell-free DNA were extracted from fresh or formalin-fixed, paraffin-embedded (FFPE) tumor samples and blood samples, respectively, according to optimized protocols. A minimum of 50 ng of DNA is required for NGS library construction. DNA was profiled using commercially available capture-based targeted sequencing panels targeting 8, 56, or 168 cancer-related genes (Burning Rock Biotech, Guangzhou, China). The genes were captured and sequenced with paired-end reads and target sequencing coverage of 1000X for tissue samples and 10,000X for plasma samples, which can detect point mutations, insertion-deletions, copy number variations, and gene rearrangement/fusions. Sequencing data were analyzed using proprietary computational algorithms that enabled variant calls to be accurately detected

by discriminating sequencing artifacts from true mutations.

Immunohistochemistry

FFPE tissue sections were utilized for IHC analysis using Ventana ALK (Clone D5F3) CDx assay kit (Roche, Arizona, USA) according to the manufacturer's directions on automated equipment. Two pathologists independently evaluated the results and discussed discordant cases until a consensus on inter-observer concordance was reached. ALK positivity was defined as the appearance of strong granular cytoplasmic staining in representative areas away from necrotic and hemorrhagic cellular materials regardless of the percentage of positive areas.

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (version 5.01). Chi-squared test was used to assess patient characteristics. Kaplan–Meier method was used to estimate PFS, while comparisons were estimated by Log-rank test. $P < 0.05$ was considered to be statistically significant.

Results

Comparison of Detection Assays and First-Line Treatment in 472 ALK-Positive NSCLCs

Of the 9440 patients diagnosed with NSCLC, ALK rearrangements were detected from 472 patients using NGS and/or IHC, resulting in an ALK mutation rate of 5%. Of them, ALK status was evaluated by only NGS in 58.9% (278/472) of the patients, only IHC in 15.9% (75/472) patients, and by both NGS and IHC in 25.2% (119/472) of the patients (Figure 1). The ALK detection concordance rate between NGS and IHC was 77.4% (92/119). Among the 27 discordant cases, 23 was NGS-positive but IHC-negative, while 4 cases were opposite, with NGS-negative and IHC-positive (Table 1). Details of first-line treatment for these 472 patients with ALK-positive NSCLC are illustrated in Figure 1. In our study, except for traditional therapies, 69 patients received first-line 2nd-generation ALK inhibitors, including alectinib (n=59), lorlatinib (n=1), ceritinib (n=1), AP26113 (n=3), and TQB3139 clinical trial (n=5).

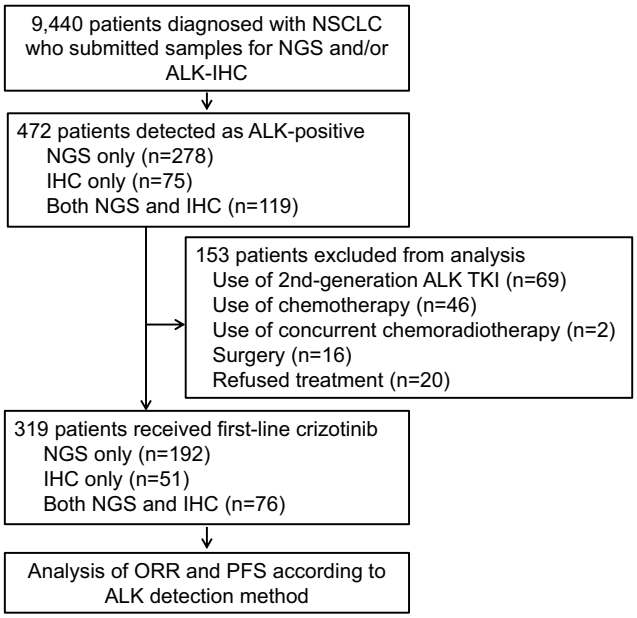

Figure 1 Flow diagram of the study design.
Abbreviations: ORR, objective response rate; PFS, progression-free survival time; NGS, next-generation sequencing; IHC, immunohistochemistry.

Clinical Characteristics of 319 First-Line Crizotinib-Treated Patients

Of the 472 patients with ALK-positive NSCLC included in our study, 319 patients received crizotinib as first-line treatment and were evaluable for treatment efficacy. The overall ORR was 86.5% (276/319) (Table 2). The median age was 51.3 years old (range 23–82 years). The cohort comprised of 58.7% (187/319) females and 72.7% (232/319) never-smokers. Histologic examination characterized a majority of patients with adenocarcinoma (96.0%, 306/319), 4 patients with adenosquamous carcinoma, and 9 patients had unspecified histology. Among the 319 crizotinib-treated ALK-positive patients, ALK status was evaluated with only NGS in 60.2% (192/319) of the patients, with only IHC in

Table 1 Distribution of Patients According to ALK Detection Method (N= 119)

		NGS		Total	Concordance Rate
		Positive	Negative		
IHC	Positive	92 (77.4%)	4 (3.3%)	96 (80.7%)	77.4%
	Negative	23 (19.3%)	0	23 (19.3%)	

Table 2 Clinical Characteristics of the 319 Patients with ALK-Positive NSCLC Who Received First-Line Crizotinib Therapy

	Patients, N(%)				
Characteristic	**All (n=319)**	**NGS Only (n=192)**	**IHC Only (n=51)**	**NGS and IHC (n=76)**	***P***
Median age, years (range)	51.3 (23–82)	42.2 (28–75)	50.7 (23–68)	44.5 (33–82)	
Sex					
Male	132 (41.3%)	73 (38.0%)	30 (58.8%)	29 (38.1%)	0.125
Female	187 (58.7%)	119 (62.0%)	21 (41.2%)	47 (61.9%)	
Smoking history					
Never smoker	232 (72.7%)	144 (75.0%)	30 (58.8%)	58 (76.3%)	0.158
Former smoker	87 (27.3%)	48 (25.0%)	21 (41.2%)	18 (23.7%)	
Pathology					
Adenocarcinoma	306 (96.0%)	185 (96.3%)	51 (100%)	70 (92.1%)	0.826
Adenosquamous carcinoma	4 (1.2%)	2 (1.1%)	0 (0%)	2 (2.6%)	
Not otherwise specified	9 (2.8%)	5 (2.6%)	0 (0%)	4 (5.3%)	
ECOG performance status					
0–1	284 (89.1%)	176 (91.6%)	47 (92.1%)	61 (80.2%)	0.100
≥2	35 (10.9%)	16 (8.4%)	4 (7.9%)	15 (19.8%)	
Brain metastasis					
Yes	57 (17.8%)	31 (16.1%)	7 (13.7%)	19 (25.0%)	0.403
No	262 (82.1%)	161 (83.9%)	44 (86.3%)	57 (75.0%)	
Stage					
IIIa/IIIb	25 (7.8%)	13 (6.7%)	5 (9.8%)	7 (9.2%)	0.810
IV	294 (92.2%)	179 (93.3%)	46 (90.2%)	69 (90.8%)	
Best response					
Complete Response	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0.978
Partial Response	276 (86.5%)	162 (84.3%)	46 (90.1%)	67 (88.1%)	
Stable Disease	32 (10.2%)	21 (10.9%)	5 (9.9%)	6 (9.2%)	
Progressive Disease	9 (2.8%)	6 (3.1%)	0 (0%)	3 (2.7%)	
NA	2 (0.6%)	2 (1.7%)	0 (0%)	0 (0%)	
Objective Response Rate	86.5%	84.3%	90.1%	88.1%	
Disease Control Rate	96.7%	95.2%	100%	97.3%	

16.0% (51/319), and with both NGS and IHC in 23.8% (76/319) of the patients. The baseline characteristics showed no difference among these groups in terms of age, sex, smoking history, ECOG PS, pathological classification, and baseline brain metastasis as summarized in Table 2.

Comparison of First-Line Crizotinib Efficacy of the Cohort

We further compared the efficacy of first-line crizotinib according to the ALK detection method. Patients whose ALK status was evaluated using NGS only, IHC only, or both NGS and IHC at diagnosis had no statistically different ORR and PFS. The ORR was 84.3% for patients in NGS only group, 90.1% for IHC only group, and 88.1% for patients tested with both NGS and IHC (p=0.95, Figure 2).

Regardless of ALK detection method, patients with ALK-positive NSCLC who received first-line crizotinib (n=319) had significantly better PFS as compared with those who received initial chemotherapy (n=46, 12.0 months vs 6.8 months; p<0.0001; Figure 3A). Meanwhile, the median PFS (mPFS) had not been reached by the patients who received second-generation ALK inhibitors but have a trend of longer PFS (n=69, undefined vs 12.0 months; p<0.0001; undefined vs 6.8 months; p<0.0001; Figure 3A). The mPFS were not statistically

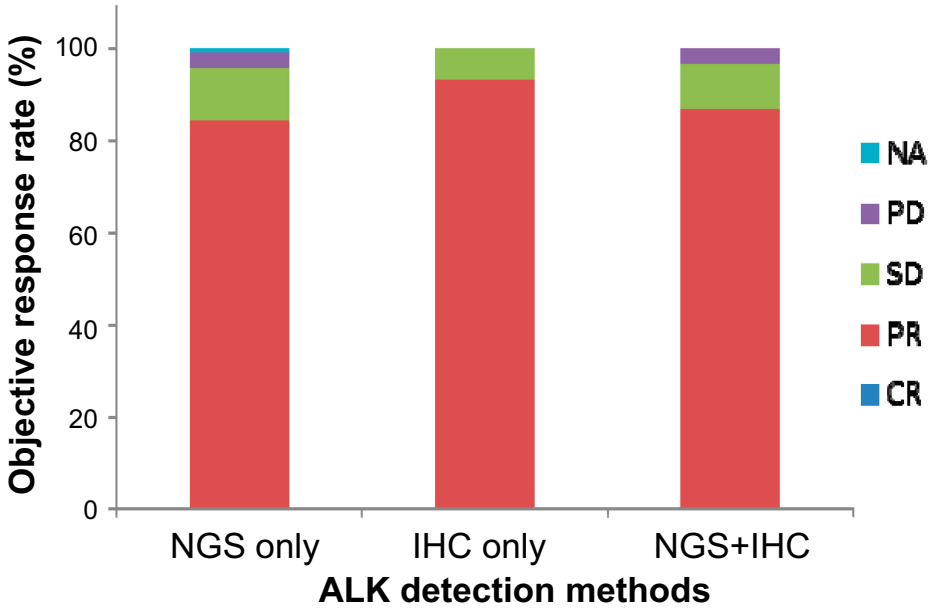

Figure 2 Comparison of ORR based on ALK detection methods of 319 patients with ALK-positive NSCLC who received first-line crizotinib treatment. X-axis represents the ALK detection methods with NGS only, IHC only, or both NGS and IHC. Y-axis denotes the ORR.
Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NA, not assessed.

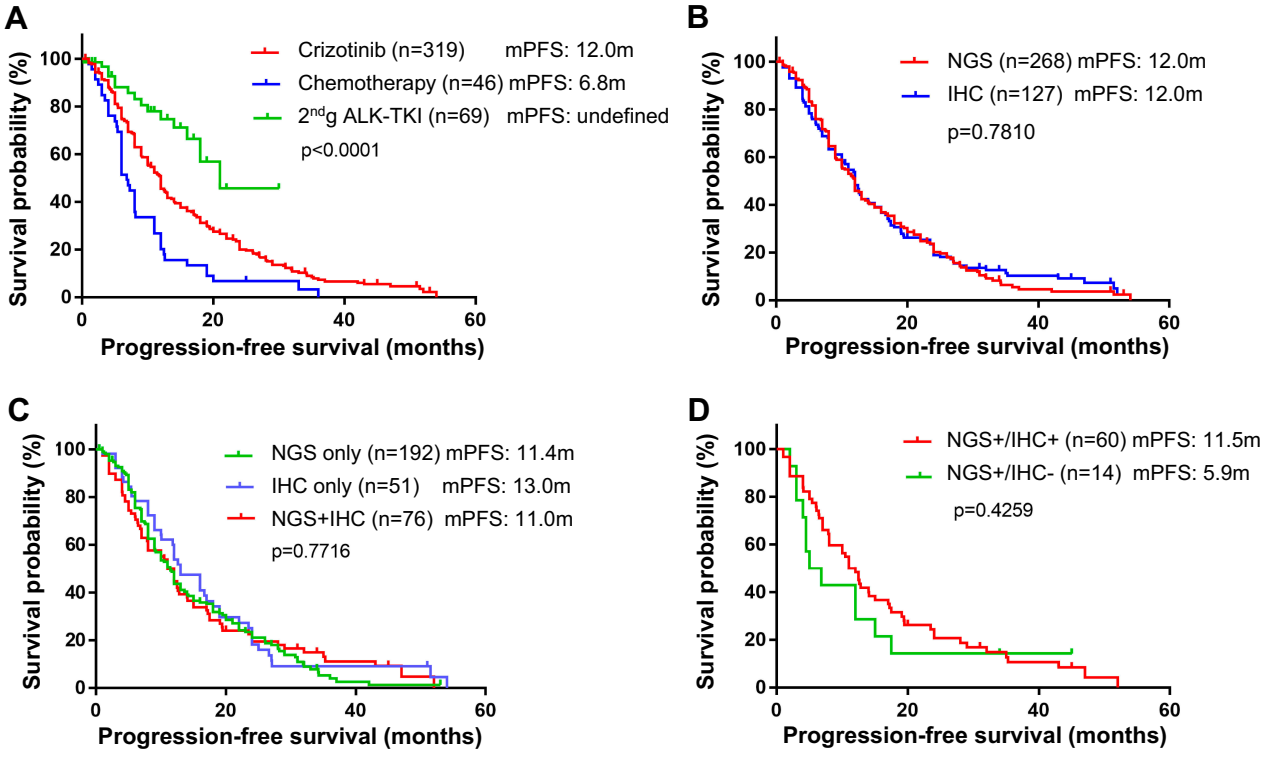

Figure 3 Progression-free survival of all 472 patients according to (**A**) the first-line treatment received by the patients, including crizotinib (n=319), chemotherapy (n=46), or second-generation ALK inhibitor (n=69) and (**B**) the ALK detection methods used for ALK status assessment, including NGS (n=268) and IHC (n=127). (**C**) Comparison of PFS among 319 patients who received first-line crizotinib who underwent different ALK detection methods such as NGS only (n=192), IHC only (n=51), and both NGS and IHC (n=76). (**D**) Comparison of PFS between the patients with ALK-positive status on both NGS and IHC (n=60) and those who had discordant NGS and IHC results (n=14) who received first-line crizotinib and underwent both NGS and IHC-based ALK assessment.

Table 3 Objective Response Rates to First-Line Crizotinib of the 76 Patients with *ALK*-Positive NSCLC According to ALK Detection Method

Response Rates		Patients, N(%)			
	All, N(%) (n=76)	**NGS+/IHC+ (n=60)**	**NGS+/IHC- (n=14)**	**NGS-/IHC+ (n=2)**	***P***
Complete Response	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0.8208
Partial Response	66 (86.8%)	51 (85.0%)	13 (92.8%)	2 (100%)	
Stable Disease	7 (9.2%)	7 (11.6%)	0 (0%)	0 (0%)	
Progressive Disease	3 (4.0%)	2 (3.4%)	1 (97.2%)	0 (0%)	
Objective Response Rates	86.8%	85.4%	92.8%	100%	
Disease Control Rates	96.0%	96.6%	92.8%	100%	
Median PFS (months)	11.0m	11.5m	5.9m	undefined	0.4259

different between patients who were assessed with NGS (n=268) and those who were assessed with IHC (n=127; 12.0 months vs 12.0 months; p=0.78; Figure 3B). Among the patients who received first-line crizotinib therapy, the mPFS of the patients in NGS only group was 11.4 months, 13.0 months for IHC only group, and 11.0 months for those who had both NGS and IHC testing (p=0.77, Figure 3C).

Treatment Efficacy of First-Line Crizotinib in 76 Patients with Both NGS and IHC results

Of the 119 patients who had both NGS and IHC-based ALK testing, only 76 patients received first-line crizotinib. A majority had concordant results between NGS and IHC (NGS+/IHC+; 80.0%, 60/76). Among the 16 patients who had discordant results between NGS- and IHC-based methods of ALK assessment, 14 were evaluated as ALK positive with NGS, but negative with IHC (NGS+/IHC-). Only 2 patients were negative for NGS but positive for IHC (NGS-/IHC+). Both patients with NGS-/IHC+ achieved partial response to crizotinib until the last follow-up date (Table 3). The ORR of the patients with NGS+/IHC+ ALK was 85.4% and was not statistically different from those with NGS+/IHC- ALK with ORR of 92.8% (p=0.82, Table 3). Meanwhile, the disease control rate was 96.6% for those with NGS+/IHC+ ALK and 92.8% for those with NGS+/IHC-ALK. As compared to patients with NGS +/IHC+ ALK, those with NGS+/IHC-ALK had a trend of shorter PFS, although statistical significance was not reached (5.9 months vs 11.5 months, p=0.43, Figure 3D).

Discussion

To the best of our knowledge, our study is one of the few studies with the largest sample size that compared two routinely used methods for ALK detection in clinical practice, and evaluated their value in predicting therapeutic response with first-line crizotinib. We demonstrated the concordance between NGS and IHC methods in ALK detection and revealed that both NGS- and IHC-based methods are reliable in detecting ALK to characterize the eligibility of the patients for crizotinib therapy. However, based on the trend of longer PFS, ALK-positive NSCLCs detected by both NGS and IHC have a better response to crizotinib. Furthermore, simultaneous assessment with both NGS- and IHC-baseds method of ALK detection can avoid false-negative cases to a large extent, which could ensure the accurate identification of the patients with ALK-positive NSCLC who can benefit from crizotinib.

FISH remains as the gold standard method for detecting ALK rearrangements.[25] Based on the 2018 guidelines, ALK IHC is considered an acceptable alternative to FISH.[23,26,27] Numerous studies have compared the reliability of ALK detection using different methods, including IHC, FISH, and RT-PCR.[23,26,28-32] However, only a few studies investigated the reliability of NGS as compared with other traditional detection methods.[18–23] In this study, we compared NGS - a method that has become routinely used in clinical oncology due to its multiplex-ability- with IHC - an established method that is routinely used for the molecular diagnosis of ALK in the clinical setting. Based on our data, ALK detection using NGS and IHC was 77.3% to 78.9% concordant, which is slightly lower than a previous report that demonstrated an 87.3% concordance rate.[19] Among the

16 patients with discordant ALK status from our cohort, 87.5% (14/16) were NGS-positive but IHC-negative, indicating that NGS was more sensitive in detecting ALK rearrangements. This observed discordance might be due to the difference in ALK alteration being detected by NGS and IHC. NGS detects genomic rearrangements involving ALK similarly to FISH, but with the simultaneous detection of other genomic alterations, while IHC detects ALK protein overexpression possibly contributed by ALK rearrangement. Hence, IHC might not be able to identify the subset of patients who harbor ALK rearrangement but did not result in ALK protein overexpression. From our cohort, approximately 18.4% (14/76) of the patients had the risk of being missed if only IHC was used for ALK detection. Of these 14 patients, 13 responded from first-line crizotinib therapy. In contrast, only 2 patients were IHC-positive but NGS-negative, but both exhibited response to crizotinib, indicating a lower false-negative rate for NGS testing. These data indicate the advantage of using NGS in identifying patients with ALK-positive NSCLC who could benefit from first-line crizotinib therapy.

The ORR to first-line crizotinib was similar between the patients who were IHC-negative and IHC-positive; however, patients with both NGS and IHC ALK-positive results had a trend of more durable response.

There were several limitations within our study including the retrospective nature of this work, and the inclusion of patients enrolled only at a single center that could potentially introduce patient selection bias. Moreover, results from FISH detection, which is considered as the gold standard for ALK assessment was not included in this comparative study. The main purpose of our study is mainly to evaluate the practical value of NGS and IHC for screening the patients initially diagnosed with advanced-stage NSCLC for eligibility to receive first-line crizotinib therapy. In clinical practice, FISH is not the preferred method due to the rigorous data interpretation and strict sample and assay requirements,[9,33,34] therefore we consider that it does not affect the conclusion of our study.

In conclusion, we demonstrate the high concordance in ALK status detected using IHC and NGS from patients with ALK-positive advanced NSCLC who benefitted from first-line crizotinib. Although NGS could detect more patients with ALK-positive tumors who could benefit from crizotinib treatment, ALK status determined by both NGS and IHC were partially predictive for longer PFS. Optimally, both diagnostic approaches should be simultaneously used to screen for patients with ALK-positive advanced NSCLC for crizotinib eligibility in clinical practice.

Clinical Practice Points

- ALK status is routinely assessed with Ventana immunohistochemistry (IHC). Next-generation sequencing (NGS) has been increasingly used in clinical oncology practice; however, the clinical implication of ALK rearrangements detected by NGS still remains unclear.
- Our results demonstrate that ALK status detected by NGS and/or IHC is reliable in identifying patients with ALK-positive NSCLC who will benefit from ALK-TKI therapy.
- ALK detected using IHC and NGS was highly concordant (78.9%).
- Crizotinib significantly prolongs PFS compared with chemotherapy in ALK-positive NSCLC.
- Patients with NGS-positive/IHC-negative ALK status respond to first-line crizotinib therapy, suggesting that NGS-based ALK detection method can predict response to crizotinib.
- Although both NGS and IHC are able to identify patients who are eligible for ALK inhibitor therapy, the simultaneous use of both diagnostic methods in the assessment of ALK status is the most optimal approach to maximize the number of patients who could clinically benefit from ALK inhibitor therapy.

Data Sharing Statement

All the data generated during this study are included in this published article. The datasets analyzed during the current study are available from the corresponding authors (Nong Yang or Zhenxing Wang) on reasonable request.

Ethics Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Hunan Cancer Hospital and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standard. Institutional review board approval was obtained from Hunan Cancer Hospital IRB Committee (approval number: 2017YQ-225).

Patient Informed Consent

Written informed consent was obtained from all the patients prior to inclusion to the study.

Author Contributions

All authors contributed towards data analysis, drafting and critically revising the paper, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work. Liang Zeng, Yizhi Li and Qinqin Xu should be considered as co first authors

Funding

This work received financial support in the form of grants from the Natural Science Foundation of China (81760529), Natural Science Foundation of Hunan Province (2018RS3106, 2018SK50901, 2019TJ-N04 to Yongchang Zhang, 2019JJ50357 to Wenjuan Jiang, 2019SK4010 and 2020JJ3025), Natural Science Foundation of Qinghai Province (2017-ZJ-942Q), CAS "Light of West China Program", Jiangsu Province Science and Technology Plan Project (BE2018660 to Zhenxin Wang), and Natural Science Foundation of Jiangsu Higher Education Institutions of China (19KJA24000 to Zhenxin Wang). The funding agencies had no role in the study design, data collection, analysis, interpretation, manuscript writing and decision to submit the article for publication.

Disclosure

Analyn Lizaso and Xinru Mao are employed by Burning Rock Biotech. The authors report no other possible conflicts of interest in this work.

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