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Comparison of ALK detection by FISH, IHC and NGS to predict benefit from crizotinib in advanced non-small-cell lung cancer

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ABSTRACT

Purpose: Anaplastic lymphoma kinase (ALK) is now a validated kinase target in non-small cell lung cancer (NSCLC). We implemented three ALK laboratory methodologies: fluorescence in situ hybridization (FISH), immunohistochemistry (IHC) and next-generation sequencing (NGS) to detect EML4-ALK fusions and compared the predictive value for Crizotinib efficacy in ALK-positive patients.

Methods: 55 ALK positive patients confirmed by at least one method were enrolled in the present study, of whom 45 cases were assessed by FISH, IHC and NGS concurrently, and another 10 cases only received IHC and NGS assessment for ALK status.

Results: IHC presented the uppermost positive rate (94.5%), followed by NGS (92.7%) and FISH(82.4%), among which IHC and NGS had the highest concordance rate of 87.3%. No difference was detected in ORR, DCR and PFS of ALK positive cases defined in three groups. Notably, NGS positive patients were correlated with a higher DCR and longer PFS compared to NGS negative cases (P = 0.02 and P = 0.09), while FISH and IHC status were not distinguishing in predicting the outcome of Crizotinib. TP53 concurrent mutation might reduce responsiveness to Crizotinib and worsen prognosis in ALK-rearranged NSCLC.

Conclusion: FISH present a certain false-negative rate although considered the gold standard. Ventana-D5F3 IHC is qualified as a screening tool, while NGS positive may predict clinical benefit of Crizotinib more accurately, allowing efficient test for specific variants and concurrent genomic alterations.

1. Introduction

Lung cancer is the most frequent cause of cancer-related mortality worldwide [1]. Over the last decade, molecularly targeted therapy based on genomic classification of patients has revolutionized the therapeutic landscape in advanced non-small cell lung cancer (NSCLC) [2]. Tyrosine kinase inhibitors antagonizing key oncogenic alterations, such as activating mutations and chromosomal rearrangements, have remarkably prolonged the survival of defined subsets of patients [3,4].

The therapeutic strategy of ALK-rearranged NSCLC is considered a paradigm for personalized oncology. First described in 2007, EML4-ALK rearrangement was the most common ALK fusion gene and had an estimated prevalence of 3–5% in NSCLC, mostly dominated by adenocarcinoma on histology [5–7]. The discovery of this driver gene led to accelerated approval of first ALK tyrosine kinase inhibitor, crizotinib, which was superior to cytotoxic chemotherapy with higher response rate and improved progression-free survival [8–10]. Other rare non-EML4 fusions,like KIF5B–ALK and TFG-ALK, have also been identified as oncogenic events in NSCLC, indicating therapeutic potential of ALK inhibition by analogy with EML4-ALK fusions, though their exact frequency and clinical significance have not been fully characterized [11–13].

ALK status is clinically pivotal for determining eligibility for ALK-directed targeted therapy. As current gold standard for ALK rearrangements detection, Vysis ALK Break Apart fluorescence in situ hybridization (FISH) kit (Abbott Molecular, Abbott Park, IL) was approved by U.S. Food and Drug Administration (FDA) in 2011 [14]. The Ventana ALK (D5F3) IHC assay, another routine diagnostic method using a D5F3 rabbit monoclonal primary antibody to detect aberrant ALK protein expression, has also been authorized by U.S. FDA as a companion diagnosis of Ceritinib [15]. Nevertheless, the best laboratory method to determine ALK status in lung cancer remains

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controversial for their own limits. The advent of next-generation sequencing methods (NGS) has facilitated high-throughput molecular analysis for a mass of genes in a single test, providing a cost- and tissue-efficient alternative to simultaneously detect gene alterations including copy number alterations, deletions, insertions, rearrangements, and single-base substitutions [16–18]. To date, ALK assessment by NGS methods has not been clinically validated, and the concordance with traditional methods is not well established.

In this study, IHC, FISH and NGS analysis were applied in a relatively large collection of ALK-positive cases, and the specificity and sensitivity of three methods for the detection of ALK-fusion in patients were compared. In addition, we make exploratory research on whether different diagnostic methods vary in predicting response to ALK-targeted therapies and clinical outcome.

2. Methods

2.1. Patients

From January 2014 to December 2017, 55 ALK-positive patients at Zhejiang Cancer Hospital were enrolled. The inclusion criteria were: (1) pathologically confirmed advanced NSCLC with at least 1 measurable lesion; (2) ALK-positive assessed with any of the 3 methods: FISH, immunohistochemistry (IHC), and Next-generation sequencing (NGS). For all patients, ALK rearrangement was assessed before ALK-TKIs treatment. Patients' clinical and treatment information were extracted from electronic medical records and all tissues used for ALK rearrangement assay were from the Zhejiang Cancer Hospital tissue bank. The histological classification was based upon the World Health Organization Criteria (2015version) [19]. The histological subtype classification of adenocarcinoma followed the standards of IASLC/ATS/ERS. Lung cancer staging was performed according to the 7th TNM classification scheme. Study protocols were approved by the Ethical Review Community of Zhejiang Cancer Hospital. The requirement of informed consent was waived by the committee as it was a retrospective research.

2.2. FISH

All FISH tests were conducted in the department of pathology in Fudan University Shanghai Cancer Center. FISH was carried out on formalin-fixed paraffin-embedded (FFPE) sections using the Vysis ALK Break Apart FISH kit (Abbott Molecular, Abbott Park, IL) according to manufacturer's instructions. The LSI ALK 5′ probe (Spectrum Green) and the LSI ALK 3′ probe (Spectrum RED) were labeled, hybridized and evaluated along with standard controls. FISH signals were evaluated independently by two technicians who were blinded to the patient's history and histologic findings. All samples were examined by pathologists to identify tumor cell enriched areas which were marked on the underside of the slides with a diamond-tipped scribe.The percentage of tumor cells in each case was over 60%. The tumor cells were considered to be FISH-positive when there were separated green and red signals (>2 signal diameters) or individual red signals, while FISH-negative was defined as overlapping red and green signals (yellowish). The criteria for ALK status determination in sample was: At least 50 tumor cells were observed. A sample was considered positive if >25 cells out of 50 ($>25/50$ or $>50\%$) are positive. If 5–25 cells out of 50(10%–50%) are positive, the sample was considered equivocal, and the slide should be evaluated by the second reader who selected additional 50 nuclei. In this case, the sample was considered positive if more than 15 of the accumulated 100 cells have a separation signal ($\geq 15\%$).

2.3. IHC

Immunohistochemistry for ALK protein expression was performed on FFPE sections using VENTANA ALK(Clone D5F3)CDx Kit and benchmark Ultra Immunostainer (Ventana Medical Systems, Inc., Tucson, AZ, Cell Signaling Technology) according to the manufacturer's instructions. Presence of strong granular cytoplasmic staining in tumor cells (any percentage of positive tumor cells) is considered positive for ALK, while absence of strong granular cytoplasmic staining in tumor cells is considered negative for ALK.

2.4. NGS

Geneseeq Technology (Nanjing, China) was responsible for the whole NGS as a centralized clinical testing center. Briefly, genomic DNA was extracted from tumor FFPE tissues using a DNeasy Blood & Tissue Kit (Qiagen Inc., USA). The KAPA Hyper Prep Kit (Kapa Biosystems, USA) was utilized for DNA library preparation as a versatile reagent kit adapted to the Illumina platform. For hybridization enrichment, customized xGen lockdown probes (Integrated DNA Technologies, USA) were applied. The probes panel were designed to target 416 cancer-specific genes. Hybrid Capture Selection was carried out using NimbleGen SeqCap EZ Hybridization & Wash Kit (Roche Inc., USA) and Dynabeads M-270 Streptavidin (Life Technologies, USA). All procedures were conducted according to the manufacturers' protocols.

2.5. Assessment of efficacy

The objective efficacy of Crizotinib was evaluated by spiral computed tomography scans every eight weeks according to the RECIST 1.1 [20]. Progression-free survival (PFS) was defined as the time from the first medication to the first objective progression of disease or the date of death from any causes. Outpatient or telephonic follow-up was adopted, and the last follow-up time of this study was on October 1, 2018.

2.6. Statistical methods

Statistical analysis was done by GraphPad Prism 6. Comparisons between clinical features, curative effects were tested by χ^2 test or Fisher's exact test. PFS was estimated by Kaplan-Meier curve and the differences were tested by log-rank test. Linear association between the abundance of ALK-fusion and the PFS of crizotinib were tested with Spearman correlations. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Patient characteristics

Patients were included in the present study when confirmed ALK positive by at least one method. Totally, 55 ALK-positive patients with a median age of 54 years (range, 27–73 years) were evaluated. The clinicopathologic profiles of the patients were displayed in Table 1. The characteristics of the patients were consistent with those in previous studies, in which most were younger than 65 years of age, had never smoked, and had lung adenocarcinoma [21,22]. A total of 40 patients received Crizotinib treatment (21 as first-line, 19 as second-line or posterior line therapy). Six patients received Alectinib as first line therapy for entering clinical trials, and nine patients did not receive any ALK-TKIs.

3.2. ALK assessable cases

The present study included 55 ALK-positive patients, of whom 45 cases were assessed by FISH, IHC and NGS concurrently. Another 10 cases only received IHC and NGS assessment for ALK status. The profile of test result was depicted in Fig. 1. Of the 45 cases assayed by FISH, 34(75.6%) had assessable results, while 11 cases (24.4%) did not show appropriate hybridization signals (i.e., weak or no green signal or

Table 1
Clinical characteristics of patients at baseline.

Characteristic	All patients(n = 55)	
	n	%
Age, Years		
Median	54	
Range	27-73	
< 65	51	93
≥65	4	7
Sex		
Male	28	51
Female	27	49
Smoking History		
Never smoker	34	62
Ever smoker	21	38
Pathology Type		
Adenocarcinoma	50	91
Squamous	0	0
NSCLC,NOS	5	9
ECOG PS		
0–1	53	96
2–3	2	4
EGFR Status		
Wild	55	100
Mutated	0	0
ALK-TKI Usage		
Crizotinib	40	73
Alectinib	6	11
No usage	9	16
Line of Crizotinib Usage		
First	21	38
Second/posterior line	19	35

Abbreviations: NSCLCNOS, non-small cell lung cancer, not otherwise specified; ECOGEastern Cooperative Oncology Group; PSperformance status; EGFRepidermal growth factor receptor; ALKanaplastic lymphoma kinase; TKItyrosine kinase inhibitor.

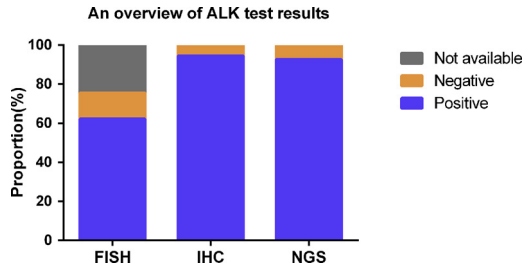

Fig. 1. An overview of ALK test results by FISH, IHC and NGS. ALK indicates anaplastic lymphoma kinase; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; NGS, next-generation sequencing. Not available means unassessable cases by FISH for technical reasons.

isolated R signal). Positive ALK fusion, determined by FISH, was identified in 82.4% of cases (28 of 34). Assessable data were obtained for IHC and NGS assays from all 55 cases, respectively. ALK protein, evaluated by IHC, was over-expressed in 94.5% of cases (52 of 55). ALK fusion gene by NGS was observed in 92.7% of cases (51 of 55).

3.3. Comparison of ALK status assessed by FISH and IHC

Among 34 malignant lung tumor samples having results for both FISH and IHC, comparison of FISH results with IHC results showed concordance between two methods in 28 of 34 cases. 27 cases were positive by both methods, and 1 case was negative by both methods. Thus, the concordance rate between FISH and IHC was 82.4%. The discrepancies were 5 cases with positive expression of ALK by IHC but not detected by FISH and one case with negative IHC expression but positive ALK fusion by FISH (Table 2).

Table 2
Assignment of ALK Status: FISH Versus IHC.

	FISH +	FISH-	Total, n	Accordance
IHC +	27	5	32	0.82
IHC-	1	1	2	
Total	28	6	34	

Abbreviations: FISH, flouresence in situ hybridization; IHC, Immunohistochemistry.

Table 3
Assignment of ALK Status: FISH Versus NGS.

	FISH +	FISH-	Total, n	Accordance
NGS +	28	6	34	0.82
NGS-	0	0	0	
Total	28	6	34	

Abbreviations: FISH, flouresence in situ hybridization; NGS, Next Generation Sequencing.

3.4. Comparison of ALK status assessed by FISH and NGS

From the 34 malignant lung tumor samples having results for both FISH and NGS, the comparison between results of FISH and NGS methods revealed agreement in 28 of 34 cases. All 28 cases were positive by both methods. These results showed concordance of 82.4% between FISH and NGS. All 6 discordant samples were NGS positive but FISH negative (Table 3).

3.5. Comparison of ALK status assessed by IHC and NGS

55 lung tumor specimens were assessed by both IHC and NGS. Of these, 48 cases were concordant. The concordance rate between IHC and NGS was 87.3%. 48 cases were positive and no case was negative by both methods. In 7 discordant samples, 3 cases were positive by NGS but without ALK over-expression by IHC while 4 cases were negative by NGS but had ALK protein over-expression by IHC (Table 4).

3.6. Comparison of FISH, IHC, and NGS to predict clinical efficacy of Crizotinib

A total of 40 patients received Crizotinib treatment, and all cases have been evaluated by IHC and NGS for ALK status. Among them, 21 cases had assessable FISH results, while 9 cases failed to be judged as positive or negative. The results detected by each method were displayed in Fig. 2. The Venn diagram revealed that IHC group included the most solely positive cases (4 cases), while NGS group had 4 solely negative cases. The therapeutic evaluation and corresponding number of patients were: 1 patient with CR, 27 patients with PR, 9 patients with SD, and 3 patients with PD.

We compared the PFS of ALK positive cases defined by each method in three groups, and the results demonstrated no significant difference as shown in Fig. 3A (mPFS: FISH 8.8 m, IHC 10.3 m, NGS 11.1 m). The ORR of FISH+, IHC + and NGS + groups were similar as 70.6%, 68.4% and 75%, and the DCR were respectively 94.1%, 94.7%,97.2%. Similarly, we compared the ORR, DCR and PFS of ALK negative cases in

Table 4
Assignment of ALK Status: NGS Versus IHC.

	NGS +	NGS-	Total, n	Accordance
IHC +	48	4	52	0.87
IHC-	3	0	3	
Total	51	4	55	

Abbreviations: NGS, Next Generation Sequencing; IHC,Immunohistochemistry.

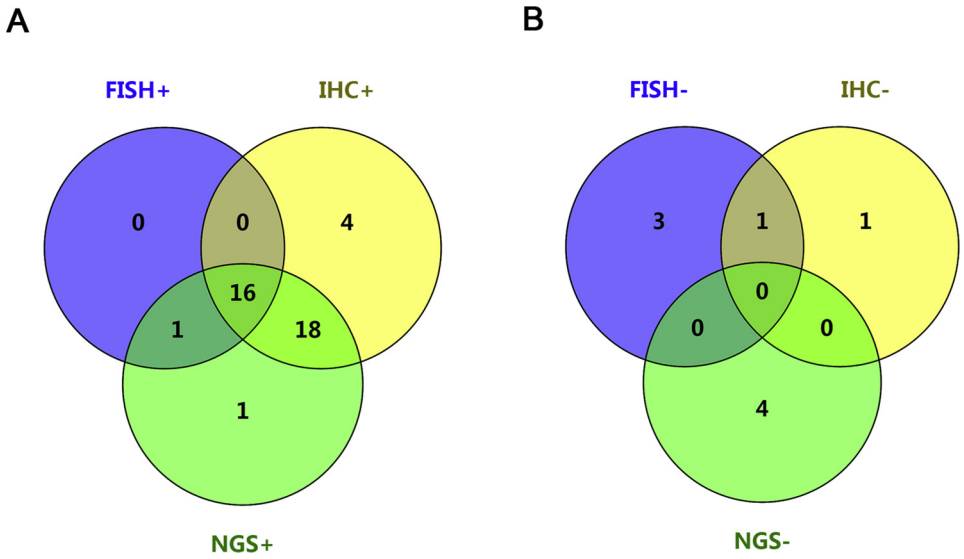

Fig. 2. The Venn diagram of ALK positive(A) and ALK negative(B) cases detected by three methodologies in 40 patients who received Crizotinib. All cases have been evaluated by IHC and NGS for ALK status. Among them, 21 cases had assessable FISH results, while 9 cases failed to be judged as positive or negative.

three groups with reference to the triple positive group (FISH +,IHC + and NGS+). The ORR of FISH-, IHC-, NGS-, and Tri_Pos groups were 75%, 100%, 25% and 68.8%, and the DCR were respectively 100%, 100%, 50% and 81.3%. According to the results in Fig. 3B (mPFS: FISH 14.8 m, IHC 11.7 m, NGS 4.6 m, Tri_Pos 8.3 m), people who were diagnosed as NGS negative actually had a inferior PFS than the reference group, but FISH/IHC negative groups did not display the trend, giving a clue that patients who were NGS negative may not be optimal candidates for Crizotinib.

Further, we explored the dissimilitude of clinical efficacy between ALK + and ALK- patients in each group when accepting Crizotinib and found no significant differences between ALK status and PFS in three groups (FISH, P = 0.75; IHC, P = 0.93; NGS, P = 0.09), as depicted in Fig. 4A, except that NGS-confirmed ALK positive patients tended to have a better PFS than those patients who were NGS negative but considered ALK positive by other methods. A higher ORR and DCR were also displayed in NGS positive cases compared with NGS negative cases (P = 0.07 and P = 0.02), while there were no statistic difference in ORR or DCR in FISH or IHC groups (Fig. 4B).

3.7. The correlation between the treatment lines and the efficacy of Crizotinib

Among 40 patients, the ORR of Crizotinib used in the first line was 76.2%, and the DCR was 90.5%, while the ORR of Crizotinib used in the second line and above was 63.2%, and the DCR was 94.7%. Comparing the ORR and DCR of crizotinib between the first and second/posterior line, the results showed no significant difference (P = 0.49; P = 1). The median PFS for the overall population of Crizotinib was 11.0 m (95% CI, 8.2 m–13.7 m). There was a marginal statistical difference in mPFS between the first-line and the second/posterior line treatment as 12.9 m (95% CI, 10.1 m–15.6 m) versus 9.0 m (95% CI, 6.4 m–11.6 m) (Z = 3.58, P = 0.06) (Fig. 5A).

3.8. Association between ALK variants and clinical efficacy of Crizotinib

We underwent next generation sequencing in all 40 cases who received Crizotinib, and ALK-positive were detected in 36 cases, of which EML4-ALK occupies the dominant, accounting for 80.6% (29 of 36). We also detected 7 cases with 11 rare ALK variants (4 cases had dual ALK fusion sub-types), namely ALK:intron19-GPR39&MIR663B concomitant with ALK:intron19-NFU1:intron1, HIP1-ALK, LHFPL4-ALK, AFAP1L1

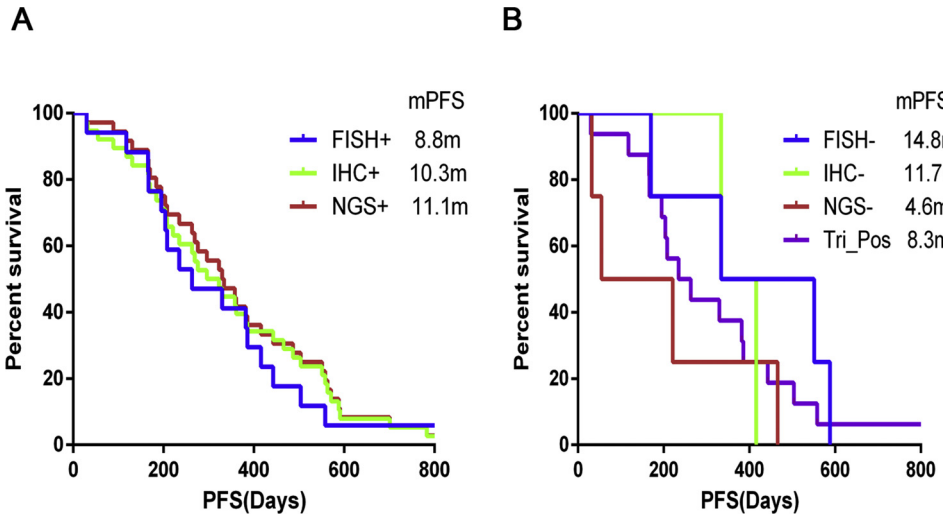

Fig. 3. The comparison of PFS in ALK positive and ALK negative cases receiving Crizotinib defined by three methods. (A) In ALK positive cases defined by each method in three groups, the results demonstrated no significant difference in mPFS. (B) The PFS of ALK negative cases in three groups were compared with reference to the triple positive group (FISH +,IHC + and NGS+) with mPFS. Abbreviations: Tri_Pos, triple positive.

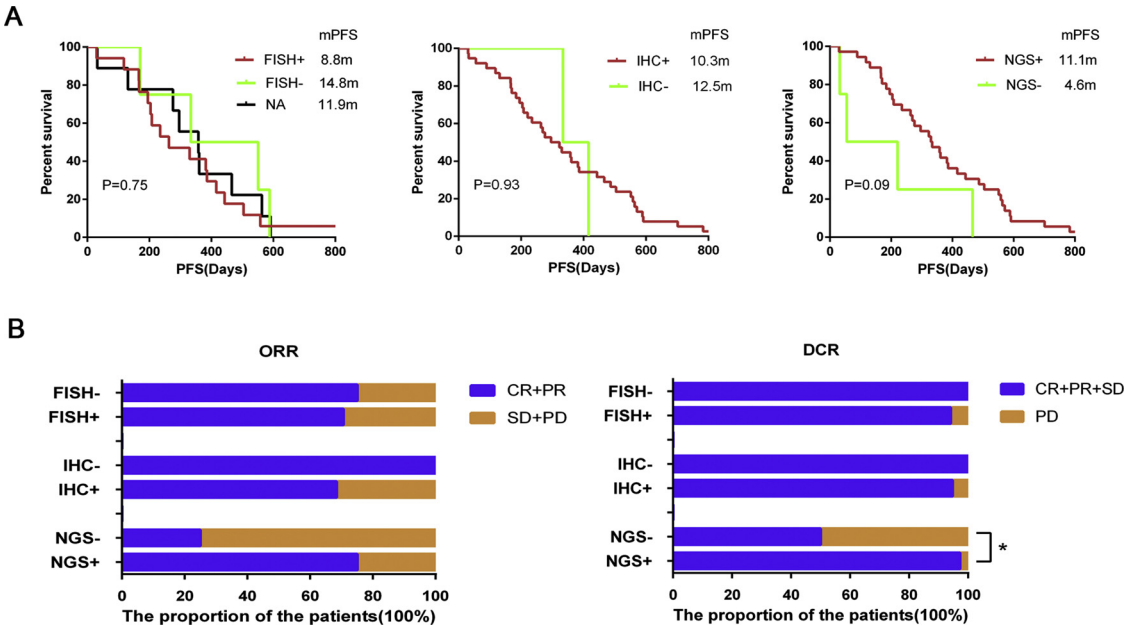

Fig. 4. The comparison of clinical efficacy between ALK + and ALK- positive patients in each group. (A) The comparison of PFS between ALK + and ALK- positive patients in each group. (B) The comparison of ORR and DCR between ALK + and ALK- positive patients in each group. Abbreviations: ORR, overall response rate; DCR, disease control rate. * P < 0.05.

intron2-ALK intron19, NAA30&AP5M1-ALK concomitant with ALK:intron19-GPR155&WIPF1, INPP5D:intron5-ALK:intron3 concomitant with KCNJ15&DSCR10-ALK : intron19, ALK:intron1-ANO1:intron5 concomitant with IGSF11&TCSC7-ALK:exon14. The PFS was compared between EML4-ALK variants and rare ALK variants and there was no statistic difference between variant sub-types of the fusion genes as 11.0 m (95% CI, 6.2 m–15.8 m) versus 11.1 m (95% CI, 10.2 m–12.1 m) with P = 0.16 (Fig. 5B). For the concurrent mutation gene existed in 29 cases, TP53 was the most frequent gene, accounting for 45%(13/29), followed by RB1 happened in 3 of 29 cases. The ORR of TP53 gene wild group was higher compared to mutation group patients (83% versus 61%, P = 0.24). When comparing the PFS in ALK fusion without TP53 mutation cases to ALK fusion with TP53 mutation cases, we detected a prolonged PFS in the former group as 12.7 m (95%CI, 7.3 m–18.2 m) versus 6.9 m(95%CI,3.8 m–10.0 m), and the difference approached statistic significance(P = 0.13) (Fig. 5C).

4. Discussion

Application of reliable screening methods for gene rearrangement detection is vital in selecting patients eligible for tumor targeted therapy. Optimally, the method should be reproducible and reliable, and validated for its clinical and biologic accuracy. In this study, we compared FISH, IHC and NGS for the detection of ALK fusion and explored the predictive value of clinical outcome including ORR, DCR and PFS by three methods in a comparatively large cohort of ALK-positive cases. As a result, IHC presented the uppermost positive rate (94.5%), followed by NGS (92.7%) and FISH (82.4%), among which IHC and NGS had the highest concordance rate of 87.3%. No difference was detected in ORR, DCR and PFS of ALK positive cases defined in three groups. Notably, NGS positive patients tended to have a higher DCR and longer PFS compared to NGS negative cases, while FISH and IHC status were not distinguishing in predicting the outcome of Crizotinib. In particular, TP53 concurrent mutation might reduce responsiveness to Crizotinib and worsen prognosis in ALK-rearranged NSCLC.

Hitherto, consensus has not been achieved regarding the best detection approaches for ALK fusion gene. Detection of ALK rearrangement by FISH is currently the gold standard, which has been validated in a series of clinical trials [9,23]. Nevertheless, in our study, 11 of 45 (24.4%) cases evaluated by FISH had inconclusive results for technical reasons like weak or no green signal detected or isolated R signal, implying FISH assays could be technically challenging and complicated to

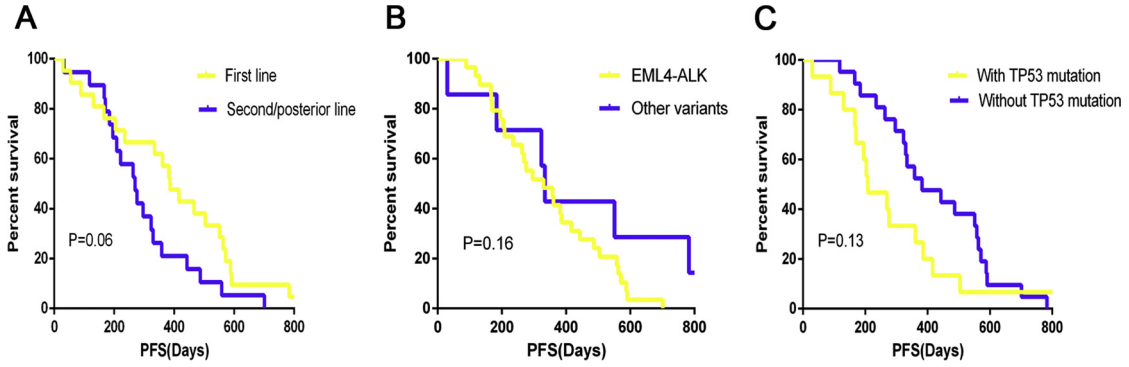

Fig. 5. (A)The correlation between the treatment line and the efficacy of Crizotinib. The result showed there was a marginal difference in mPFS between the first-line and the second/posterior line treatment. (B) The association between ALK variant sub-types and clinical efficacy of Crizotinib. (C)The comparison of PFS in ALK fusion without TP53 mutation cases to ALK fusion with TP53 mutation cases.

interpreting, probably due to the subtlety of the emitted signal and the topographic location of these genes in the chromosome [24]. The possibility of signal decay after long-term tissue block storage might account for it [25]. Secondly, patients with advanced NSCLC usually only provide small biopsy. Sometimes it is difficult to ensure that more than 50 lung cancer cells are interpreted. Thirdly, intratumoral heterogeneity, which was reported to coexist with histologic heterogeneity in both single-driver and ALK/EGFR coaltered LADCs, may result in the discrepance. Altered oncogenic drivers in spatially separated subclones of the same tumor may be different [26]. Among nine FISH-inconclusive cases using Crizotinib, 9/9 were IHC ALK + and 7/9 were NGS ALK+, and the overwhelming majority (8/9) achieved response to Crizotinib, indicating an omission of potential targeted therapy population by FISH. While in 21 FISH-defined cases, 3 of 4 FISH negative cases were proved PR to the targeted therapy and 1 was evaluated as SD. It was in part similar to the study of Ali et al., which identified 11 FISH ALK negative cases in 31 NGS ALK positive patients (35%) and found 7 of 11(64%) FISH ALK negative cases sensitive to Crizotinib [27]. Other studies that have applied NGS to confirm inconsistent IHC and FISH results also implied that FISH could deny patients' access to ALK inhibitors for a significant false-negative rate [28–31]. Hence, the combination of ALK fusion detection method would achieve higher accuracy. The borderline cut off values, as Camidge et al. confirmed that when > 4 fields were counted, the > 15% cell positivity cut point would ensure maximal sensitivity and specificity, affected the detection results from another perspective [32]. The high costs, time consuming, specific equipment, and trained personnel also make it not feasible for routine use.

The correlation between ALK IHC staining and FISH detection has been extensively reported in the literature [33,34]. Among several available ALK IHC antibodies, Clone D5F3 (Cell Signaling Technology) has been evaluated by several groups. Minca et al. concluded that IHC using the D5F3 IHC antibody was more informative than FISH, including adding more ALK-positive cases [35]. Marina et al. claimed that IHC (D5F3 antibody) provided excellent sensitivity and specificity (100% and 97.7%, respectively), suggesting a promising alternative to FISH [36]. In our study, Ventana-D5F3 IHC assay was adopted and it presented the highest positive rate (94.5%) among three methodologies. Characteristics like time-saving, accessible and economical also ensure the foundational role in ALK screening [37].

As additional biomarkers of clinical significance continue to emerge for solid tumors, the applicability and sustainability of single-gene assessment is increasingly questionable, particularly for limited biopsy material. NGS methods provide a highly suitable and cost-effective solution for addressing the evolving needs of comprehensive genotyping [38,39]. Herein, NGS was performed in all 40 cases who had received Crizotinib and we noticed that NGS positive indicated a higher DCR and longer PFS when compared with NGS negative cases. Hence, NGS might be more reliable to pick out optimal candidates for targeted therapy.

The broad scope and flexibility of NGS in specifying and detecting ALK fusions have been well documented by the public literature [40,41]. In this study, among 45 cases that had been assessed by FISH, IHC and NGS concurrently, 8 cases were ALK positive by NGS but negative for either FISH or IHC assays. Their ALK fusion types were: EML4-ALK(5 cases), EML4-ALK + ALK-DIRC3 (one case), EML4-ALK + ALK-AFAP1L1(one case) and ALK-ANO1 + IGSF11&TCSC7-ALK (one case). An AFAP1L1 intron2-ALK intron19 fusion gene with unaffected sensitivity to Crizotinib was only detected by NGS. In one FISH-/IHC + case, two novel ALK translocation partners, ANO1and IGSF11&TCSC7 were identified, highlighting the capability of NGS to detect novel translocation partners not captured by other methodologies. Additionally, the PFS was compared between EML4-ALK variants and rare ALK variants. As a result, no statistic difference was detected between variant sub-types of the fusion gene, indicating the applicability of Crizotinib in various types of ALK fusions, which, however, need further validation by studies with larger samples.

Few studies have focused on the effects of concurrent mutations with ALK fusions on Crizotinib therapy. Based on a comparatively large ALK fusion population, TP53 was found to be the most frequent gene, accounting for nearly the half. Particularly, we validated a higher ORR and a prolonged PFS in TP53 gene wild group compared to TP53 mutation group with the difference approaching statistic significance. The result was in accordance with previous findings that TP53 mutation reduced responsiveness to Crizotinib and worsened prognosis in ALK-rearranged NSCLC patients [42]. Interestingly, Li et al. reported that 5 of 8 EML4-ALK FISH negative but IHC/NGS positive patients had TP53 mutations and speculated a role of TP53 mutation in DNA chromothripsis in some ALK rearrangement events [43], just as a context-specific role in catastrophic DNA rearrangements in pediatric medulloblastoma and acute myeloid leukemia [44,45]. Therefore, NGS was advantageous in detecting concurrent mutations and investigating their relevance with testing methodologies and prognosis.

In summary, there is a moderate level of concordance among FISH, IHC and NGS in detecting ALK fusion. All three methods result in similar but not identical Kaplan-Meier plots for PFS. NGS is particularly advisable in cases where FISH and IHC results are inconclusive and NGS positive may predict clinical benefit of Crizotinib more accurately. Additionally, NGS prompt the information on different ALK fusion subtypes and concomitant mutations, thereby facilitating the exploration of molecular mechanisms underlying the complicated gene rearrangement events.

Disclosure of potential conflicts of interest

The authors have declared no conflicts of interest.

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