ORIGINAL ARTICLE

Identification and therapeutic evaluation of *ALK* rearrangements in non-small-cell lung cancer

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Abstract

This study aimed to present a comprehensive assessment of anaplastic lymphoma kinase (*ALK*) rearrangements evaluated by DNA/RNA-based next-generation sequencing (NGS) and Ventana immunohistochemistry (IHC) in patients with non-small-cell lung cancer (NSCLC) and to evaluate the therapeutic outcomes of ALK tyrosine kinase inhibitor (TKI) treatment. We investigated *ALK* gene fusions in 14,894 patients with NSCLC using Ventana IHC and NGS, including 12,533 cases detected via DNA-based NGS and 2,361 cases using RNA-based NGS. The overall percentage agreement (OPA), positive percentage agreement (PPA), and negative percentage agreement (NPA) were calculated when comparing the results between NGS and IHC. The therapeutic responses to ALK-TKIs were also evaluated. In total, 3.50% (439/12,533) of specimens were NGS ALK-positive (NGS-p) in the DNA-based NGS cohort and 3.63% (455/12,533) were IHC ALK-positive (IHC-p). The OPA of NGS was 99.60%, whereas its PPA and NPA were 92.75 and 99.86%, respectively. In the adenocarcinoma (ADC) subcohort, the PPA was 95.69%. In the RNA-based NGS cohort, 2.20% (52/2,361) of specimens were NGS-p and 2.63% (62/2,361) were IHC-p. The OPA of NGS was 99.49%; its PPA and NPA were 82.26 and 99.96%, respectively. Thirteen patients with discordant results received ALK-TKI treatment. In the seven NGS-p/IHC-negative (IHC-n) patients, the overall response rate (ORR) was 85.4% (6/7) and the disease control rate (DCR) was 100%. In the six NGS-negative/IHC-p patients, the ORR was 66.7% (4/6) and the DCR was 100%. In summary, a high concordance of *ALK* gene fusion detected via NGS and IHC was observed in this study. DNA-based NGS had a higher OPA, PPA, and PPA in the ADC subcohort, whereas RNA-based NGS had a higher NPA. Overall, the results suggest that the combination of NGS and IHC can improve the accuracy of *ALK* fusion detection; hence, a result determination algorithm for clinical detection of *ALK* gene fusion was also proposed.

Keywords: ALK; NGS; Ventana IHC; NSCLC; ALK-TKI

Introduction

Anaplastic lymphoma kinase (*ALK*) gene rearrangements have been reported in 3–5% of non-small-cell lung cancer (NSCLC) cases [1]. *ALK* gene rearrangements generally encode fusion oncoproteins with the ALK kinase domain constitutively activating downstream cell signalling pathways including the PI3K, JAK/STAT, and RAS/MEK/ERK pathways [2]. ALK tyrosine kinase inhibitors (TKIs), such as crizotinib, ceritinib, and alectinib, can effectively block ALK activity in patients with NSCLC with *ALK* rearrangements [3].

In addition, different *ALK* fusion partners or variants may have different ALK activities that could affect an individual's response to ALK-TKIs [4,5]. The canonical *EML4-ALK* fusion is the most common *ALK* fusion variant. In addition to *EML4*, other fusion partners of *ALK* have been identified, such as *KIF5B* and *KLC1*, among others. Therefore, it is necessary to use next-generation sequencing (NGS) in clinical practice to provide specific information on fusion partners of *ALK* and other genes. With the application of NGS, more *ALK* fusion partners and variants have been identified. To date, more than 15 *EML4-ALK* fusion

variants have been reported, including *EML4-ALK* variant 1 (E13:A20), variant 2 (E20:A20), variant 3 (E6:A20), variant 4 (E14:ins11del49A20), and variant 5 (E2:A20).

Several approaches have been used to identify *ALK* rearrangements, including fluorescence *in situ* hybridisation (FISH), reverse transcription-polymerase chain reaction (RT-PCR), immunohistochemistry (IHC), and NGS. NGS includes DNA-based and RNA-based NGS. The US FDA (Food and Drug Administration)-approved Ventana ALK (D5F3) antibodies (Ventana Medical Systems, Inc., Tucson, AZ, USA) have been used to routinely detect the presence of the ALK protein because of their high sensitivity, specificity, cost-effectiveness, and convenience of operation [6]. DNA-based NGS detects *ALK* rearrangements at the DNA level and can detect both reported and novel *ALK* rearrangements using a designed probe that hybridises on certain regions of the *ALK* gene. On the other hand, RNA-based NGS detects *ALK* fusions at the RNA level. Amplification-based RNA-based NGS assays concerning gene-specific primer pairs have a rapid turnaround time; however, they can only detect fusions with known partners because such assays only utilise sequence-specific primer pairs to enrich targeted regions. Other RNA-based methods, such as anchored multiplex polymerase chain reaction [7], are specifically designed to detect gene fusions through ligation-mediated amplification and can identify novel fusion events without known fusion partners or breakpoints. The results of ALK detection using these detection methods at different levels have been generally shown to be consistent. However, inconsistent results arise in some cases because of the complexity of the transcription and translation mechanisms. Multiple studies have compared the results of *ALK* evaluations using different approaches [8,9]. In our previous study, we validated a portion of Ventana ALK-positive cases obtained from Ventana using capture-based NGS in patients with NSCLC [10]. However, reports on ALK evaluation with Ventana IHC and DNA-based or RNA-based NGS in a large cohort of patients are limited.

In this study, we retrospectively identified ALK status using Ventana IHC and DNA-based NGS or RNA-based NGS in 14,894 patients with NSCLC to estimate the concordance of different methods. The efficacy of ALK-TKI therapy in patients with discordant results was also evaluated. This way, the effectiveness of the combination of NGS and IHC in the clinical testing of *ALK* gene fusion was analysed, and some recommendations for clinical testing were made.

Materials and methods

Patients

A total of 14,894 NSCLC specimens were collected in this study, including 10,211 surgical specimens and 4,683 small biopsy or cell block specimens (transbronchial biopsies, endobronchial ultrasound transbronchial needle aspirations, and pleural effusion cell blocks) from Shanghai Chest Hospital, which were simultaneously detected via NGS and Ventana IHC between 2017 and 2021. The histotypes of each specimen were diagnosed by two experienced pathologists and were classified according to the 2015 World Health Organization (WHO) classification scheme. The clinicopathological features of the specimens are presented in Table 1. Informed consent was obtained from all subjects, and the study was approved by the Ethics Committee of the Shanghai Chest Hospital.

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and national research committees and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Genomic DNA/RNA extraction

Genomic DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissues using a QIAamp DNA FFPE Tissue Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. DNA was quantified using a Qubit 3.0 system (Life Technologies, Carlsbad, CA, USA). Total RNA was extracted from FFPE tissues using a QIAamp RNA FFPE Tissue Kit (Qiagen GmbH) and was quantified using Qubit 3.0.

DNA-based NGS

In total, 12,533 samples were analysed using the DNA-based NGS method. Library preparation for 68 lung cancer-related genes, covering 0.345 Mb of the human genome, was performed using a DNA capture-based NGS panel (Burning Rock Biotech, Guangzhou, PR China) (supplementary material, Table S1). A total of 100–200 ng of genomic DNA from FFPE tissues was fragmented using Covaris M220 ultrasonicator (Covaris Woburn, MA, USA). An enriched library was then used to hybridise with the capture probes. The amount and size of the library were assessed using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Sequencing was performed using a NextSeq 550

Table 1. Relationship between *ALK* fusion status and clinicopathological features in patients with NSCLC

Features	DNA-based NGS				RNA-based NGS			
	All	ALK+	ALK−	*P* value	All	ALK+	ALK−	*P* value
Age				<0.001				<0.001
Mean	60.6	54.1	60.8		60.4	52.7	60.6	
Median	62	54	63		62	52	62	
Range	14–90	19–84	14–90		18–87	26–74	18–87	
Sex				0.131				0.184
Male	6439	210	6229		1123	20	1103	
Female	6094	229	5865		1238	32	1206	
Specimen type				<0.001				0.001
Surgical	8174	239	7935		2037	37	2000	
Biopsies/cell blocks	4359	200	4159		324	15	309	
Histology				<0.001				<0.001
ADC	10 638	424	10 214		2089	52	2037	
SCC	1530	5	1525		221	0	221	
ASC	91	5	86		13	0	13	
NSCLC-NOS	200	5	195		22	0	22	
Other NSCLCs	74	0	74		16	0	16	

ASC, adenosquamous carcinoma; NSCLC-NOS, NSCLC not otherwise specified; other NSCLC cases include LCC (large-cell carcinoma), SC (sarcomatoid carcinoma), ACC (adenoid cystic carcinoma), and LELC (lymphoepithelioma-like carcinoma).

sequencer (Illumina, Inc., San Diego, CA, USA). NGS sequence reads were aligned to the hg19 version of the human genome using BWA-MEM 0.7.10. The data processing methods employed here have been described previously [11].

RNA-based NGS

In addition, 2,361 other samples were analysed through a custom-designed assay using Ion AmpliSeq targeted sequencing technology (Thermo Fisher Scientific, Carlsbad, CA, USA). This assay includes both DNA and RNA panels in parallel, enabling the detection of mutations and fusions of certain driver genes, including the gene fusion of *ALK*, *ROS1*, *RET*, and *NTRK1* (supplementary material, Table S2). Ten nanograms of RNA were reverse-transcribed to complementary DNA (cDNA) using a SuperScript VILO cDNA Synthesis kit (Thermo Fisher Scientific), and targets were amplified using a multiplex primer pool. The barcode adapters were ligated and normalised to a concentration of 100 pmol/l. Eight equimolar RNA libraries were pooled before template preparation and enrichment using Ion OneTouch2 and ES (Thermo Fisher Scientific). Sequencing was performed using an Ion S5 system (Thermo Fisher Scientific).

Sequencing data were first processed using Ion Torrent Suite version 5.2.0 (Thermo Fisher Scientific) for reference mapping and base calling, during which validation-defined QC specifications were used as acceptance criteria. Quality filters were used at the amplicon level to remove counts below the threshold

for detection and at the base-pair level for low-quality variant calls. Ion Reporter Software (Thermo Fisher Scientific) was used to detect fusion with QC metrics.

Ventana IHC

All 14,894 samples were analysed using Ventana IHC for ALK protein expression. IHC was performed on 3.5-μm-thick FFPE specimens using the Ventana anti-ALK (D5F3) antibodies (Ventana Medical Systems, Inc.), together with the Optiview DAB IHC Detection Kit and OptiView Amplification Kit (Ventana Medical Systems, Inc.) on a Benchmark XT stainer, following the manufacturer's instructions.

A dichotomous system (positive or negative) was used to evaluate staining results. ALK positivity was defined as the presence of robust cytoplasmic staining in neoplastic cells. ALK negativity was defined as the absence of robust cytoplasmic staining in tumour cells, wherein the matched positive control was stained. Moreover, staining of areas of well-differentiated keratinisation in squamous cell carcinoma (SCC) was excluded. Two experienced pathologists reviewed all ALK IHC slides.

However, in the real world, some cases with weak and diffuse cytoplasmic staining or those with only a few tumour cells with cytoplasmic staining are difficult to identify. In this study, based on our experience, cases were interpreted as positive with strong (3+) cytoplasmic staining in any percentage of tumour cells or moderate (2+) cytoplasmic staining in ≥5% of tumour cells, atypical positive with moderate (2+)

cytoplasmic staining in <5% of tumour cells, atypical negative with weak (1+) cytoplasmic staining in ≥5% of tumour cells, and negative with weak (1+) cytoplasmic staining in <5% of tumour cells or without any cytoplasmic staining. Moreover, atypical positive and atypical negative cases were classified as positive and negative in the statistical analysis process, respectively.

Fluorescence *in situ* hybridisation

For some samples with inconsistent results for RNA-based NGS and Ventana IHC, FISH for *ALK* rearrangements was performed on 3.5-μm-thick FFPE specimens using a break-apart probe (Vysis LSI ALK Dual Colour, Break Apart Rearrangement Probe, Abbott Molecular, Abbott Park, IL, USA) according to the manufacturer's instructions. ALK positivity was evaluated in cases where more than 15% of the tumour cells showed a split red and green signal and/or an isolated red signal.

Reverse transcription–polymerase chain reaction

For some samples with inconsistent results for DNA-based NGS and Ventana IHC, RT-PCR for *ALK* fusion was performed using an *EML4-ALK* fusion gene detection kit (Amoydx, Xiamen, PR China) according to the manufacturer's instructions.

Evaluation of therapeutic responses to ALK-TKIs

Some patients with concordant and discordant ALK results between NGS and Ventana IHC received first- or second-line ALK-TKI treatments. Their therapeutic responses were evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. Progression-free survival (PFS) was measured from the first medication to objective tumour progression or death. The last follow-up time of this study was 23 November 2021.

Statistical analysis

Statistical analyses were performed using SPSS®, version 18.0 (IBM, Armonk, NY, USA). Chi-squared tests were used to determine the relationships between the NGS results and the clinicopathological features of the patients. The overall percentage agreement (OPA), positive percentage agreement (PPA), and negative percentage agreement (NPA) were calculated by comparing the results of Ventana IHC with those obtained via NGS. OPA = [(number of both NGS-p/IHC-p and NGS-n/IHC-n specimens)/(total number of specimens)] × 100%, PPA = [(number of NGS-p/IHC-p specimens)/(number of IHC-p specimens)] × 100%, NPA = [(number of NGS-n/IHC-n specimens)/(number of IHC-n specimen)] × 100%.

Results

DNA-based NGS

In total, 12,533 specimens were detected using DNA-based NGS that passed quality control, and 3.50% (439/12,533) were found to be ALK-positive and carried *ALK* rearrangements. The clinicopathological features of all samples are shown in Table 1. In brief, the difference in ALK positivity rate was statistically significant with respect to age, specimen type, and histotypes, but not in sex.

The types of *ALK* rearrangements are summarised in Figure 1A, wherein 89.5% of specimens (393/439) had *EML4-ALK* rearrangements. Among *EML4-ALK* rearrangements, variant 3 (E6:A20), variant 1 (E13:A20), and variant 2 (E20:A20) were the three most common types, accounting for 77.4% of the total *ALK* rearrangements. Among the 46 non-*EML4 ALK* rearrangements, 13 were canonical *ALK* rearrangements, including 7 *KIF5B-ALK* cases, 3 *KLC1-ALK* cases, and 3 *GCC2-ALK* cases. The remaining 33 non-*EML4 ALK* rearrangements were rare and noncanonical.

RNA-based NGS

In total, 2,361 specimens were analysed via RNA-based NGS, and their results passed quality control. Moreover, 2.20% (52/2,361) were identified as ALK-positive and harboured an *ALK* fusion variant. The clinicopathological features of all samples analysed via RNA-based NGS are shown in Table 1. Similar to samples evaluated using DNA-based NGS, *ALK* fusion was also more frequent in younger patients, small biopsies, and samples that were diagnosed as adenocarcinoma. There was no significant difference in ALK positivity between sexes.

The types of *ALK* fusion variants are summarised in Figure 1B, among which 96.1% (50/52) had *EML4-ALK* rearrangements. Among the *EML4-ALK* fusions, variant 1 (E13:A20), variant 3 (E6:A20), and variant 2 (E20:A20) were the three most common types, accounting for 88.4% of the total *ALK* fusions. The two non-*EML4 ALK* fusions comprised one *KIF5B-ALK* and one *KLC1-ALK* fusion.

Ventana IHC

In the DNA-based NGS cohort, 3.63% (455/12,533) were identified as Ventana IHC ALK-positive.
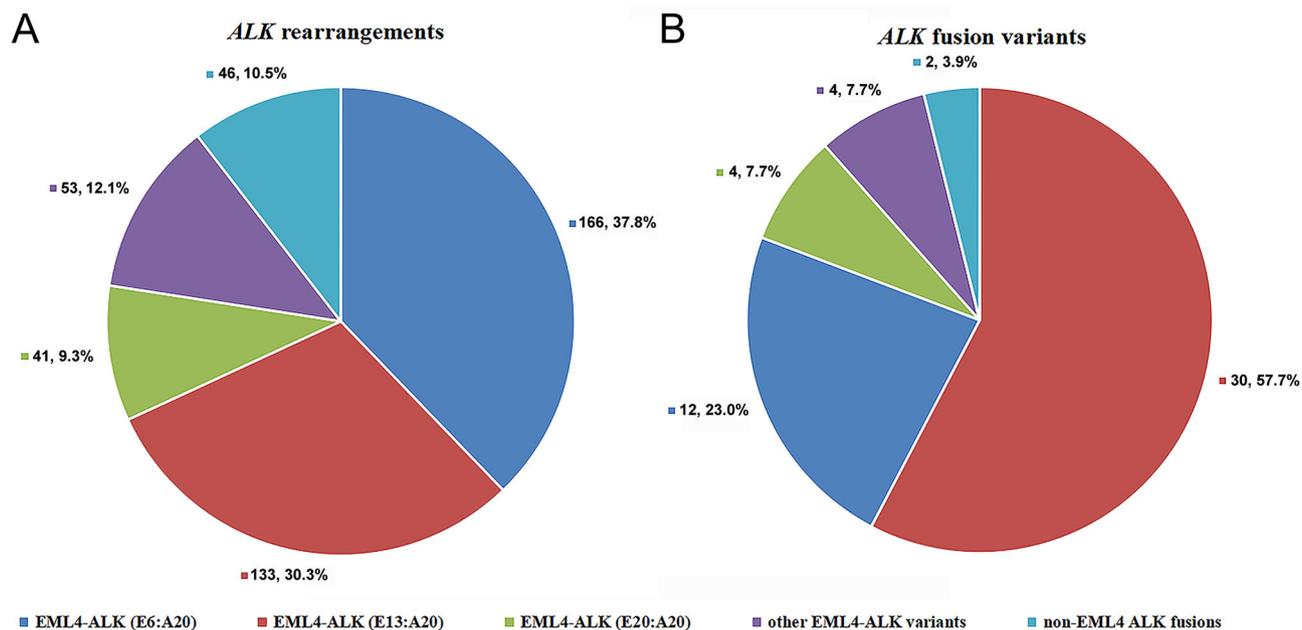
Figure 1. Frequencies of (A) different *ALK* rearrangements as detected using DNA-based NGS and (B) *ALK* fusion variants detected using RNA-based NGS.

Moreover, the Ventana IHC results included 15 atypical positive results and 1 atypical negative result. Among the 16 samples with atypical results, 13 with atypical positive results were SCC samples, while the rest were all ADC) samples. In atypical-positive SCC cases, only cytoplasmic staining was considered, regardless of the staining of the intercellular bridges or cell membranes. On the other hand, in the RNA-based NGS cohort, 2.63% (62/2,361) were identified as ALK-positive, and all of the results were typical.

All 16 cases with atypical Ventana IHC results were found in the DNA-based NGS-tested cohort. Two of them were NGS-positive, among which one was atypical IHC-positive and the other was atypical IHC-negative. Moreover, both were diagnosed with ADC. The remaining 14 cases were atypical-positive and NGS-negative. Among them, 1 was ADC, while the remaining 13 were SCC cases.

Comparison of the results between DNA-based NGS and Ventana IHC

When comparing the results of DNA-based NGS with the results of Ventana IHC, the OPA was 99.60%, while the PPA and NPA were 92.75 and 99.86%, respectively. In addition, the PPA was 95.69% in the ADC subcohort (Table 2).

To confirm the results obtained at a different level, RT-PCR was performed to re-evaluate the samples with discordant results. Of the 17 samples that were NGS-p/IHC-n (NGS-positive and IHC-negative), which were all diagnosed as ADC, 16 were RT-PCR-positive, including 1 sample with atypical-negative Ventana IHC results (Figure 2E); the remaining 1 sample was RT-PCR-negative (Figure 3). Of the 33 samples that were NGS-n/IHC-p (NGS-negative and Ventana IHC-positive), 19 were ADC samples, among

Table 2. Comparison of the results of DNA-based NGS, RNA-based NGS, and IHC

| | IHC | | | | | | |
	Positive	Negative	Total	OPA (%)	PPA (%)	PPA of ADC (%)	NPA (%)
DNA-based NGS							
Positive	422	17		99.60	92.75	95.69	99.86
Negative	33	12,061	12,533				
RNA-based NGS							
Positive	51	1		99.49	82.26	82.26	99.96
Negative	11	2,298	2,361				

which 18 were typical Ventana IHC-positive and RT-PCR-positive, while the remaining 1 sample was atypical Ventana IHC-positive and RT-PCR-negative (Figure 2C). The other 14 samples with NGS-n/IHC-p were diagnosed as SCC, among which 1 sample was typical Ventana IHC-positive and RT-PCR-positive, and the remaining 13 were atypical Ventana IHC-positive and RT-PCR-negative (Figure 2A,B).

It is worth mentioning that one of the samples with consistent positive results was an adenocarcinoma case with an atypical-positive Ventana IHC result (Figure 2D).

In addition, the lone NGS-p/IHC-n case, which was diagnosed as adenocarcinoma, was ALK-negative via IHC, but was *ALK*-positive as detected using NGS, with a *FAM114A1-ALK* (F4:A20) rearrangement involving the 5′ portion of *FAM114A1* to the 3′ portion of *ALK*, which was predicted to produce an out-of-frame transcript. This case was negative for *ALK* rearrangement as evaluated via FISH, RT-PCR, and RNA-based NGS. However, *ALK* gene amplification in this sample was detected using FISH (Figure 3).

Comparison of the results between RNA-based NGS and Ventana IHC

Comparing the results of RNA-based NGS with the results of Ventana IHC, the OPA was 99.49%, whereas the PPA and NPA were 82.26 and 99.96%, respectively. As all the positive samples were ADC samples, the PPA of ADC was 82.26% (Table 2).

FISH was carried out to re-evaluate the samples with discordant results at the DNA level. All re-evaluated samples turned out to be FISH-positive, regardless of being NGS-n/IHC-p or NGS-p/IHC-n.
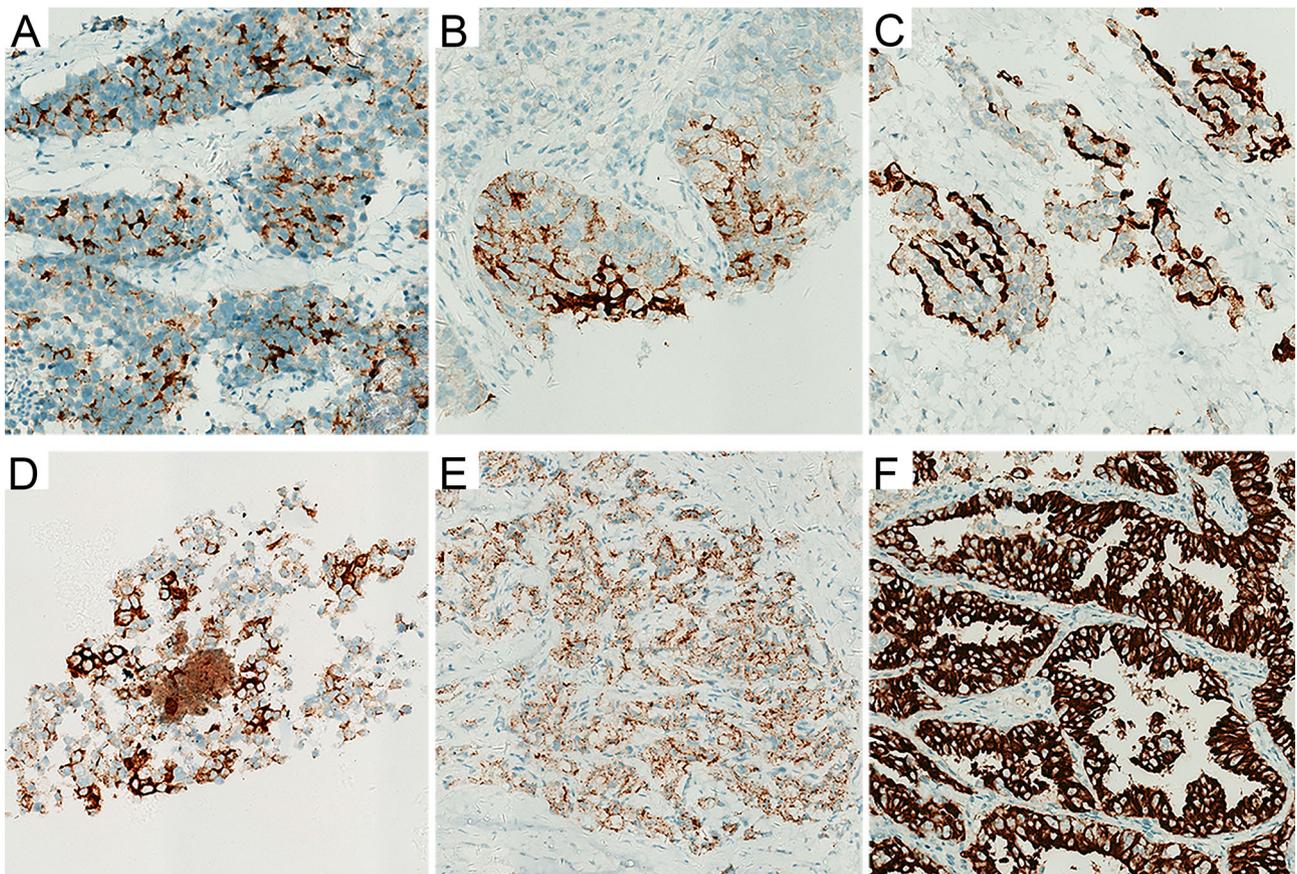

Figure 2. Ventana IHC results of several representative cases. (A and B) Two SCC cases with atypical positive results with Ventana IHC and that were both NGS-n/RT-PCR-n (NGS-negative and RT-PCR-negative). (C and D) Two ADC cases with atypical positive results with Ventana IHC. Case C was NGS-n/RT-PCR-n, while case D was NGS-p/RT-PCR-p (NGS-positive and RT-PCR- positive). (E) An ADC case with an atypical negative result from Ventana IHC and that was NGS-p/RT-PCR-p. (F) An ADC case with a typical Ventana IHC result and that was NGS-positive. Original magnification, ×200.
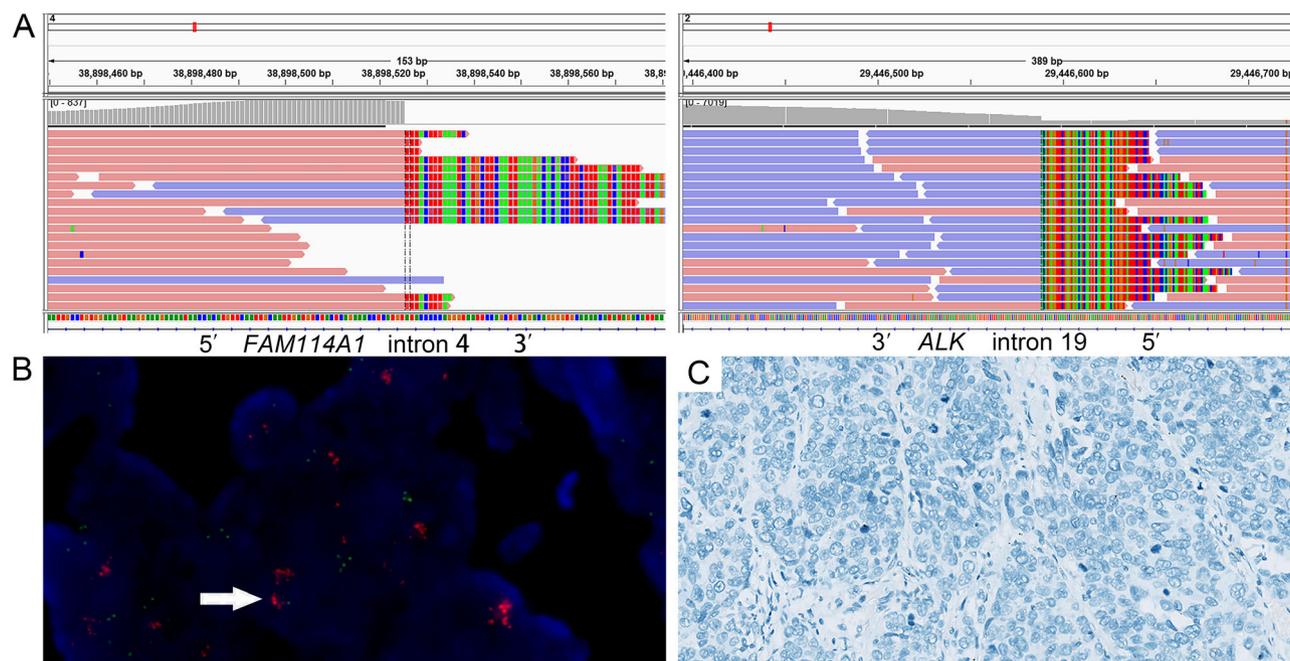
Figure 3. Results from the case that was DNA-based NGS-positive/IHC-negative/RT-PCR-negative. (A) Integrative Genomics Viewer (IGV) screenshot of *FAM114A1-ALK* rearrangement. (B) FISH detection of the amplification of the kinase domain of *ALK* as represented by the red signals (white arrow). (C) Negative result of Ventana IHC.

Evaluation of ALK-TKI treatment

In total, 103 patients with concordant positive results on NGS and Ventana IHC (NGS-p/IHC-p) received first-line crizotinib treatment. Of these, 1 had a complete response, 73 had a partial response, 26 had stable disease, and 3 had progressive disease (PD). Therefore, the overall response rate (ORR) was 71.84% (74/103), and the disease control rate (DCR) was 97.09% (100/103).

Furthermore, 13 patients with discordant NGS and Ventana IHC results received ALK-TKI treatment (Table 3). Among the seven NGS-p/IHC-n patients, two received first-line crizotinib treatment, two received first-line alectinib treatment, and the other three received second-line alectinib treatment after first-line chemotherapy. The ORR of this cohort was 85.4% (6/7) and the DCR was 100%. One patient

Table 3. Therapeutic response to ALK-TKIs of the patients with inconsistent results between NGS and IHC

Patient no.	Gender	Age (years)	Specimen type	Histology	NGS	IHC	TKI therapy	PFS (months)	Best response	Follow-up
Case 1	F	41	Biopsy	ADC	DNA-P	N	Alectinib/second-line	33.1+	PR	On-going
Case 2	M	28	Biopsy	ADC	DNA-P	N	Alectinib/second-line	15.4	PR	Progressed
Case 3	F	24	Biopsy	ADC	DNA-P	N	Alectinib/first-line	26.5+	SD	On-going
Case 4	F	57	Biopsy	ADC	DNA-P	N	Alectinib/first-line	16.7+	PR	On-going
Case 5	F	51	Biopsy	ADC	DNA-P	N	Alectinib/second-line	12.3+	PR	On-going
Case 6	F	69	Biopsy	ADC	DNA-P	N	Crizotinib/first-line	9.9+	PR	On-going
Case 7	F	38	Biopsy	ADC	DNA-P	N	Crizotinib/first-line	5.7+	PR	On-going
Case 8	M	79	Biopsy	SCC	DNA-N	P	Crizotinib/first-line	3.3+	SD	On-going
Case 9	M	54	Surgical	ADC	RNA-N	P	Alectinib/first-line	12.4+	PR	On-going
Case 10	M	66	Surgical	ADC	DNA-N	P	Crizotinib/second-line	8.5+	PR	On-going
Case 11	M	52	Biopsy	ADC	DNA-N	P	Crizotinib/first-line	8.7+	SD	On-going
Case 12	F	43	Biopsy	ADC	DNA-N	P	Alectinib/first-line	10.4+	PR	On-going
Case 13	F	59	Surgical	ADC	RNA-N	P	Alectinib/second-line	12.0+	PR	On-going

DNA-N, negative detected via DNA-based NGS; DNA-P, positive detected via DNA-based NGS; F, female; M, male; N, negative; P, positive; PR, partial response; RNA-N, negative detected via RNA-based NGS; SD, stable disease.

developed disease progression, and had a 15.4-month PFS, while the other six patients did not develop PD and were still in follow-up.

Among the six NGS-n/IHC-p patients, three patients received crizotinib treatment, including two as first-line and one as second-line treatment after first-line chemotherapy, and the remaining three patients received alectinib, including two as first-line and one as second-line treatment after first-line chemotherapy. The ORR of this cohort was 66.7% (4/6) and the DCR was 100%. PD was not observed in any of these patients.

Additionally, the difference in the best therapy response between the NGS-p/IHC-p, NGS-p/IHC-n, and NGS-n/IHC-p patients was not significant ($p = 0.437$).

Discussion

Although multiple studies have compared different testing methods for *ALK* fusions [10,12–14], reports on a large cohort evaluating ALK status using Ventana IHC and DNA-based/RNA-based NGS in NSCLC are limited. In this study, we identified ALK status using Ventana IHC and DNA-based or RNA-based NGS to provide recommendations for the clinical testing of *ALK* gene fusion in patients with NSCLC.

Overall, the results of Ventana IHC and NGS were highly consistent, with an OPA of >99% for both DNA-based NGS and RNA-based NGS with Ventana IHC. Several studies also reported that the results of IHC and NGS were consistent [15–17]; this study, which reported a large number of cases, could provide more data support.

However, the PPA of NGS with IHC was slightly lower, with a PPA of 92.75% for DNA-based NGS/IHC and 82.26% for RNA-based NGS/IHC. If only ADC cases were considered, the PPA of the DNA-based NGS/IHC was 95.69%. This indicated that the low PPA of DNA-based NGS/IHC was partly due to the non-ADC specimens. Moreover, among the IHC-p/DNA-based NGS-n samples, 58% (19/33) were and 94.7% (18/19) were ALK-positive, as verified via RT-PCR. In addition, 42% (14/33) were SCCs; only 7.1% (1/14) of these were ALK-positive as verified via RT-PCR, while the other 92.9% (13/14) were all atypical IHC-positive and ALK-negative as detected using RT-PCR. On the other hand, all 17 IHC-n/DNA-based NGS-p samples were ADCs, and 94.1% (16/17) were positive as detected using RT-PCR. This indicates that the positive results for ALK in ADC, both detected via IHC and NGS, were robust. The positive results of non-ADC samples (such as SCC)

detected using Ventana IHC were more likely to be atypical-positive, which might be a false positive. On the other hand, atypical IHC results for ADC should be verified using other methods. To date, only a few ALK IHC-positive cases have been reported in SCC [18]. Therefore, caution should be exercised in interpreting the positive results of non-ADC cases, and another method should be used for verification if necessary.

In the IHC/RNA-based NGS cohort, all positive results were found in ADC specimens, and no positive results were found in non-ADC specimens, regardless of using NGS or IHC, which might be due to the relatively limited number of cases involved. Therefore, the low PPA of RNA-based NGS/IHC was unrelated to interference from non-ADC specimens. One of the possible reasons for this was that RNA-based NGS could only detect known fusion partners; therefore, some fusion types were missed, and false-negative results might have occurred. Moreover, RNA-based NGS seems to detect fewer ALK-positive cases (2.2%; 52/2,361) than DNA-based NGS (3.5%; 439/12,533). One possible reason for this is mentioned above that the RNA-based NGS assay could only detect known fusions designed in the panel. Moreover, as shown in Table 1, the DNA-tested cohort had more small biopsy specimens (34.8 versus 13.7%) and fewer surgical specimens (65.2 versus 86.3%) than RNA-tested cohort. Generally, surgical specimens were obtained from patients diagnosed at an earlier stage, where the ALK positivity rate is lower [10,19]. Therefore, the cohort evaluated using RNA-based NGS, which had more surgical specimens, had a lower ALK positivity rate. Additionally, in the cohort of small biopsies diagnosed as ADC, the ALK-positive rates detected using DNA-based NGS and RNA-based NGS were 6.04% (190/3,144) and 7.11% (15/211), respectively. This difference was not statistically significant ($p = 0.532$).

Additionally, 13 patients with discordant NGS/IHC results, including NGS-p/IHC-n and NGS-n/IHC-p, were treated with ALK-TKIs, and all achieved good ORR and DCR. As 12 of them were ADCs and 1 was SCC with typical IHC positivity, these results suggest that ALK-TKI therapy can be used in patients with NSCLC with discordant NGS/IHC ALK results, especially in those with ADC and with typical IHC-positive or canonical NGS-positive results. Several studies have analysed the efficacy of ALK-TKIs in patients with different ALK results using different methods. Some studies have found that patients with IHC-p/FISH-n results who received crizotinib treatment achieved good ORR and DCR [20], while other studies have found that patients with IHC-p/FISH-n results

who received crizotinib did not have a good enough therapeutic response [21]. As all these studies involved relatively few cases, this difference in results may be a bias resulting from the smaller number of cases, which needs to be confirmed by conducting more studies.

Among the 17 cases with NGS-p/IHC-n results, only 1 was negative, as verified using RT-PCR and RNA-based NGS, in which the genomic rearrangement type was *FAM114A1-ALK*, demonstrating that this rearrangement might be a non-productive one. Generally, functional *ALK* rearrangements at the DNA level are transcribed into chimeric mRNAs and further translated into ALK fusion proteins containing the activated ALK tyrosine kinase domain [22]. Although *ALK* rearrangement was detected using DNA-based NGS in this case, no chimeric mRNA and fusion protein were expressed. This suggests that DNA-based NGS cannot completely distinguish whether the detected rearrangements were functional. Moreover, although this *FAM114A1-ALK* rearrangement was predicted to produce an out-of-frame transcript, it was not sufficient to determine that this case had no functional *ALK* fusion. In our previous studies, we found that cases harbouring non-productive/out-of-frame *ALK/RET* rearrangements always have canonical fusion transcripts at the RNA level [10,23]. On the other hand, it is a pity that this patient did not receive ALK-targeted therapy, so we were unable to evaluate the efficacy of ALK-TKI in this case.

Interestingly, we found an amplification of the 3′ portion of the *ALK* gene (red signal) in the former case using FISH, which might be a kinase domain duplication (KDD) event (Figure 3). Gallant *et al* [24] have confirmed that an *EGFR* KDD is an oncogenic driver clinically responsive to EGFR-TKIs. To the best of our knowledge, only a few *ALK* KDD cases have been reported [25], and the exact clinical response to ALK-TKIs is not well known. In this study, we found that there might be a relationship between *ALK* KDD and non-functional *ALK* genomic rearrangements, which requires further research.

In this study, the most common *ALK* rearrangement detected using DNA-based NGS was variant 3 (E6: A20), followed by variant 1 (E13:A20). The most common *ALK* fusion detected using RNA-based NGS was variant 1 (E13:A20), followed by variant 3 (E6: A20). Other studies using DNA-based [26] and RNA-based NGS [17,27,28] have yielded similar results. To some extent, this reflects the difference in *ALK* rearrangement/fusion detected between the DNA and RNA levels. Moreover, the proportion of non-*EML4 ALK* fusion types detected using DNA-based NGS was higher than that detected using RNA-based NGS. On one hand, DNA-based NGS can detect rare

rearrangement types, while the RNA-based NGS used in this study can only detect known *ALK* fusion types, of which most were *EML4-ALK* fusions. On the other hand, the rare noncanonical rearrangements detected at the DNA level were likely to be canonical *EML4-ALK* fusions at the RNA level. This has been confirmed in our previous study [10], and in another study [29], as well as in other studies showing that patients carrying rare noncanonical fusions or canonical *EML4-ALK* fusions showed similar therapeutic efficacy after receiving TKI treatment [30].

Following the results of this study and a review of the literature, we found that the results of Ventana IHC and NGS were highly consistent in detecting *ALK* fusion in NSCLC, which means that either method can effectively detect *ALK* gene fusion in most cases. Using either method alone, cases with atypical Ventana IHC results or noncanonical DNA-based NGS rearrangements should be verified using another method detecting at a different level. Nevertheless, based on the results of this study, using either method alone may yield false positives or false negatives. Therefore, a combination of different methods can be considered if economic and other conditions permit. Especially for institutions that use NGS for routine testing, the combination of Ventana IHC and NGS is recommended, which can greatly improve the accuracy of *ALK* fusion detection. Our recommendations for the implementation of the combination of NGS and IHC for the detection of *ALK* fusion genes and the result determination algorithm for NSCLC are summarised in Figure 4. In short, three main points that need to be emphasised. First, cases harbouring all *ALK* fusion variants detected via RNA-based NGS and canonical *ALK* rearrangements detected via DNA-based NGS carried *ALK* gene fusions, regardless of the IHC results. Second, ADC cases with typical positive Ventana IHC results had confirmed *ALK* gene fusions, regardless of the NGS results. Third, in cases other than the above two situations, another method of verification at a different level is required. In this case, discordant results between DNA-based NGS and IHC should be verified via RT-PCR, and discordant results between RNA-based NGS and IHC should be verified via FISH. The ALK status of the samples was determined based on the results of the verification method. It should be pointed out that, owing to the limitations of all these methods, it is still possible to have false-negative or false-positive samples, although this is highly unlikely.

In addition, considering the cost and sample amount limitations, we did not perform DNA-based and RNA-based NGS simultaneously on the same cohort, which
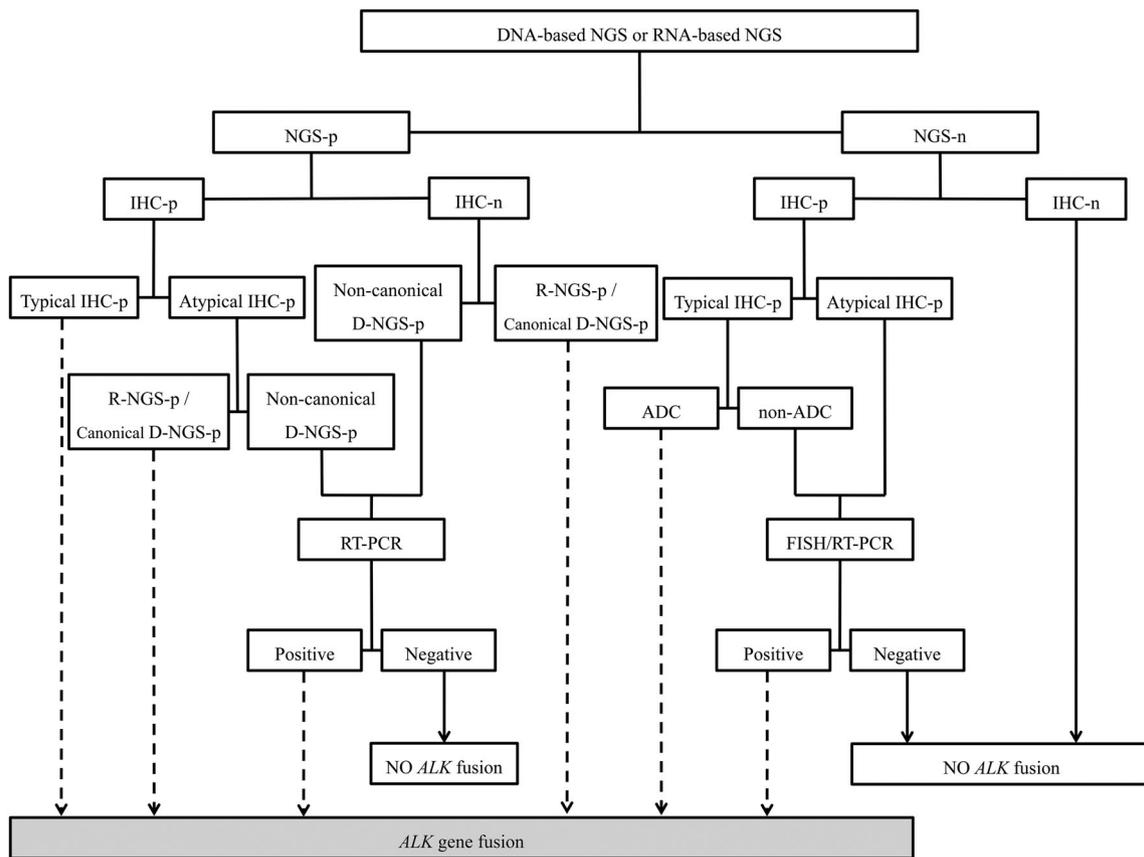
Figure 4. Recommendations for the implementation of the combination of NGS and Ventana IHC for the detection of *ALK* fusion genes and the result determination algorithm in NSCLC. Canonical D-NGS-p, ALK rearrangements such as *EML4–ALK*, *KIF5B–ALK*, *KLC1–ALK*, and *GCC2–ALK* detected by DNA-based NGS; D-NGS, DNA-based NGS; IHC, Ventana ALK D5F3; –n, negative; noncanonical D-NGS-p, rare *ALK* rearrangements other than those previously mentioned; –p, positive; R-NGS, RNA-based NGS.

might be a limitation of this study. We chose to perform RT-PCR to verify discrepant IHC/DNA-based NGS results instead of RNA-based NGS. As this would miss fusions involving partners other than *EML4*, this was also a limitation of this study.

In conclusion, a high concordance of *ALK* gene fusion detected via NGS and Ventana IHC was observed in this study. Compared with IHC, DNA-based NGS yielded a higher OPA, PPA, and PPA in ADC, whereas RNA-based NGS yielded a higher NPA. Both NGS and Ventana IHC can effectively detect *ALK* gene fusion in NSCLC cases. At the same time, the combination of NGS and IHC can improve the accuracy of *ALK* fusion detection to a greater extent. A recommendation for the implementation of the combination of these two methods to detect *ALK* gene fusion and the result determination algorithm was proposed in this study. ALK-positive results in ADC cases, including those detected using NGS and IHC, were more robust. Atypical IHC results for ADC cases should be verified using other methods. In contrast, atypical-positive results of ALK in SCC cases detected via IHC were more likely to be false positives. Patients with discordant IHC/NGS results, especially in ADC cases, achieved good ORR and DCR when treated with ALK-TKIs. The *ALK* rearrangement variant types detected using DNA-based NGS and RNA-based NGS were somewhat different, as many noncanonical non-*EML4 ALK* rearrangements at the DNA level were possibly transcribed as canonical *EML4-ALK* fusions at the RNA level.

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Author contributions statement

RZ and LG were involved in the study design, data analysis, data interpretation and the writing of the manuscript. BZ collected information on the ALK-TKI treatments and evaluated the therapeutic response. JZ, CX, SC, JS, LZ and MY performed the experiments and collected and analysed the data. YH was involved in the study conception and design, pathology assessment and article revision. All authors were involved in writing the paper and provided final approval of the submitted and published versions.

Data availability statement

The data sets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

References

1. Gerber DE, Minna JD. ALK inhibition for non-small cell lung cancer: from discovery to therapy in record time. *Cancer Cell* 2010; **18**: 548–551.

2. Lin JJ, Riely GJ, Shaw AT. Targeting ALK: precision medicine takes on drug resistance. *Cancer Discov* 2017; **7**: 137–155.

3. Hancock L. The inhibition of anaplastic lymphoma kinase in non-small cell lung tumours with the ALK rearrangement may result in tumour shrinkage. *Thorax* 2011; **66**: 332.

4. Yoshida T, Oya Y, Tanaka K, *et al*. Differential crizotinib response duration among ALK fusion variants in ALK-positive non-small-cell lung cancer. *J Clin Oncol* 2016; **34**: 3383–3389.

5. Heuckmann JM, Balke-Want H, Malchers F, *et al*. Differential protein stability and ALK inhibitor sensitivity of EML4-ALK fusion variants. *Clin Cancer Res* 2012; **18**: 4682–4690.

6. Uruga H, Mino-Kenudson M. ALK (D5F3) CDx: an immunohistochemistry assay to identify ALK-positive NSCLC patients. *Pharmgenomics Pers Med* 2018; **11**: 147–155.

7. Zheng Z, Liebers M, Zhelyazkova B, *et al*. Anchored multiplex PCR for targeted next-generation sequencing. *Nat Med* 2014; **20**: 1479–1484.

8. Letovanec I, Finn S, Zygoura P, *et al*. Evaluation of NGS and RT-PCR methods for ALK rearrangement in European NSCLC patients: results from the European Thoracic Oncology Platform Lungscape Project. *J Thorac Oncol* 2018; **13**: 413–425.

9. Zeng L, Li Y, Xu Q, *et al*. 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. *Onco Targets Ther* 2020; **13**: 7101–7109.

10. Zhao R, Zhang J, Han Y, *et al*. Clinicopathological features of ALK expression in 9889 cases of non-small-cell lung cancer and genomic rearrangements identified by capture-based next-generation sequencing: a Chinese retrospective analysis. *Mol Diagn Ther* 2019; **23**: 395–405.

11. Zhao R, Yao F, Xiang C, *et al*. Identification of *NTRK* gene fusions in lung adenocarcinomas in the Chinese population. *J Pathol Clin Res* 2021; **7**: 375–384.

12. Lin C, Shi X, Yang S, *et al*. Comparison of ALK detection by FISH, IHC and NGS to predict benefit from crizotinib in advanced non-small-cell lung cancer. *Lung Cancer* 2019; **131**: 62–68.

13. Li W, Zhang J, Guo L, *et al*. Combinational analysis of FISH and immunohistochemistry reveals rare genomic events in ALK fusion patterns in NSCLC that responds to crizotinib treatment. *J Thorac Oncol* 2017; **12**: 94–101.

14. Scattone A, Catino A, Schirosi L, *et al*. Discordance between FISH, IHC, and NGS analysis of ALK status in advanced non-small cell lung cancer (NSCLC): a brief report of 7 cases. *Transl Oncol* 2019; **12**: 389–395.

15. Jurmeister P, Vollbrecht C, Jöhrens K, *et al*. Status quo of ALK testing in lung cancer: results of an EQA scheme based on in-situ hybridization, immunohistochemistry, and RNA/DNA sequencing. *Virchows Arch* 2021; **479**: 247–255.

16. Batra U, Nathany S, Sharma M, *et al*. IHC versus FISH versus NGS to detect ALK gene rearrangement in NSCLC: all questions answered? *J Clin Pathol* 2021; **75**: 405–409.

17. Canterbury CR, Fernandes H, Crapanzano JP, *et al*. ALK gene rearrangements in lung adenocarcinomas: concordance of immunohistochemistry, fluorescence in situ hybridization, RNA in situ hybridization, and RNA next-generation sequencing testing. *JTO Clin Res Rep* 2021; **2**: 100223.

18. Wang H, Sun L, Sang Y, *et al*. A study of ALK-positive pulmonary squamous-cell carcinoma: from diagnostic methodologies to clinical efficacy. *Lung Cancer* 2019; **130**: 135–142.

19. Xiang C, Ji C, Cai Y, *et al*. Distinct mutational features across preinvasive and invasive subtypes identified through comprehensive profiling of surgically resected lung adenocarcinoma. *Mod Pathol* 2022; **35**: 1181–1192.

20. Ma D, Wang Z, Yang L, *et al*. Responses to crizotinib in patients with ALK-positive lung adenocarcinoma who tested immunohistochemistry (IHC)-positive and fluorescence in situ hybridization (FISH)-negative. *Oncotarget* 2016; **7**: 64410–64420.

21. Thunnissen E, Lissenberg-Witte BI, van den Heuvel MM, *et al*. ALK immunohistochemistry positive, FISH negative NSCLC is infrequent, but associated with impaired survival following treatment with crizotinib. *Lung Cancer* 2019; **138**: 13–18.

22. Kerr KM, López-Ríos F. Precision medicine in NSCLC and pathology: how does ALK fit in the pathway? *Ann Oncol* 2016; **27**(Suppl 3): iii16–iii24.

23. Xiang C, Guo L, Zhao R, *et al*. Identification and validation of noncanonical RET fusions in non-small-cell lung cancer through DNA and RNA sequencing. *J Mol Diagn* 2022; **24**: 374–385.

24. Gallant J, Sheehan JH, Shaver TM, *et al*. EGFR kinase domain duplication (EGFR-KDD) is a novel oncogenic driver in lung cancer that is clinically responsive to afatinib. *Cancer Discov* 2015; **5**: 1155–1163.

25. Ikeda S, Gay L, Pavlick D, *et al*. Comprehensive genomic profiling (CGP) of 114,200 advanced cancers identifies recurrent kinase domain duplications (KDD) and novel oncogenic fusions in diverse tumor types. *Ann Oncol* 2017; **28**(Suppl 10): x1–x6.

26. Xia P, Zhang L, Li P, *et al*. Molecular characteristics and clinical outcomes of complex ALK rearrangements identified by next-generation sequencing in non-small cell lung cancers. *J Transl Med* 2021; **19**: 308.

27. Lin JJ, Zhu VW, Yoda S, *et al*. Impact of EML4-ALK variant on resistance mechanisms and clinical outcomes in ALK-positive lung cancer. *J Clin Oncol* 2018; **36**: 1199–1206.

28. Tan DS, Thomas M, Kim DW, *et al*. Genetic landscape of patients with ALK-rearranged non-small-cell lung cancer (NSCLC) and response to ceritinib in ASCEND-1 study. *Lung Cancer* 2021; **163**: 7–13.

29. Li W, Liu Y, Li W, *et al*. Intergenic breakpoints identified by DNA sequencing confound targetable kinase fusion detection in NSCLC. *J Thorac Oncol* 2020; **15**: 1223–1231.

30. Cai C, Tang Y, Li Y, *et al*. Distribution and therapeutic outcomes of intergenic sequence-ALK fusion and coexisting ALK fusions in lung adenocarcinoma patients. *Lung Cancer* 2021; **152**: 104–108.

SUPPLEMENTARY MATERIAL ONLINE

Table S1. Gene list of the DNA-based NGS

Table S2. Gene fusion variants list of the RNA-based NGS