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A study of ALK-positive pulmonary squamous-cell carcinoma: From diagnostic methodologies to clinical efficacy

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ABSTRACT

Background: High concordance has been observed between Ventana D5F3 ALK immunohistochemistry (IHC) and fluorescence in-situ hybridization (FISH) in lung adenocarcinoma (LADC). However, whether a similar conclusion can be applied to lung squamous-cell carcinoma (LSCC) has remained unclear. We therefore evaluated the ALK (anaplastic lymphoma kinase) status and the therapeutic effect of an ALK tyrosine kinase inhibitor (TKI) in IHC- or FISH-positive LSCC.

Materials and methods: A total of 2403 LSCC patients from three institutions were screened for ALK aberration by IHC. All IHC-positive cases were subjected to FISH (with an approximately equal number of negative cases as a control group) and next-generation sequencing (NGS). Clinical efficacy was evaluated for the patients who received TKI therapy.

Results: In 2403 cases of LSCC, 37 cases were identified as ALK-positive by IHC. After quality control, 28 cases were succeeded by FISH (six with insufficient tissue, three with lack of signals) and 13 by NGS (24 failed due to insufficient samples or poor DNA quality); the percentage of non-diagnostic tests was 24.3% (9/37) and 64.9% (24/37), respectively. Four cases (4/2394, 0.17%) analyzed by FISH were determined as ALK-positive. For the control group (40 ALK IHC), FISH demonstrated no samples with ALK gene fusion. The concordance between ALK IHC- and ALK FISH-positive results was 14.3% (4/28). In the 13 cases studied by NGS, two cases showed *ALK-EML4* fusion (consistent with two FISH-positive results), and two cases were interpreted as harboring an ALK-association gene mutation. Among four patients (two FISH-positive and two IHC-positive only cases) receiving TKI therapy, two patients had stable disease and the other two had progressive disease.

Conclusions: The positive concordance rate of ALK IHC and FISH in LSCC is far less than that reported for LADC. Therefore, ALK IHC detection in LSCC cannot be used as a diagnostic method for ALK rearrangement.

1. Introduction

EML4-ALK fusion represents a bona fide oncogene driver and molecular target in non-small-cell lung cancer (NSCLC), and ALK-targeted therapy has demonstrated clear clinical utility for NSCLC patients with an ALK fusion gene aberration. First reported in 2007, *EML4-ALK*

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gene rearrangement was more prevalent in adenocarcinoma patients with a prevalence of approximately 2–13% [1,2]. The recommended methods for detection of ALK rearrangement include Ventana D5F3 ALK immunohistochemistry (IHC), fluorescence in-situ hybridization (FISH) and reverse transcription polymerase chain reaction (RT-PCR), all of which have shown high concordance in lung adenocarcinoma (LADC) patients [3–7]. The IHC assay has an inherent advantage for clinical adoption because of its cost-effectiveness for mass screening compared to FISH and RT-PCR. Recently, some reports suggested that IHC was superior to FISH in selecting patients responsive to ALK-targeted therapy [8–10]. Accordingly, the updated CAP/IASLC/AMP molecular testing guideline for lung cancer recommended IHC as an equivalent alternative to FISH for ALK testing, and treatment decisions can be made when IHC results are clearly positive [11].

In contrast to LADC, ALK-rearranged lung squamous-cell carcinoma (LSCC) is rare, and no large cohort studies have been reported. The estimated prevalence of ALK-positive LSCC is 0–1.36% [7,12–15]. Moreover, it remains controversial whether LSCC patients with ALK rearrangement could benefit from ALK tyrosine kinase inhibitors (TKIs) [1,15–21]. To systematically address these questions, we reviewed 2403 LSCC patients (from three institutions) whose ALK status was tested by IHC; positive cases were confirmed by FISH and NGS, and patients who received the ALK inhibitor crizotinib were followed up.

2. Materials and methods

2.1. Specimens

Between January 2013 and December 2016, all consecutive cases of primary LSCC from three institutions were reviewed retrospectively. Out of a total of 2403 cases, 806 were from the General Hospital of Chinese People´s Liberation Army, 877 were from the Tianjin Cancer Hospital and 720 were from Peking Cancer Hospital. Histological diagnosis was confirmed with LSCC markers (CK5/6, P40 or P63) and neuroendocrine markers CD56, chromogranin A and synaptophysin, if necessary, and reviewed by two pathologists. Clinical information was extracted from medical records. Consent was obtained from all patients.

2.2. ALK immunohistochemistry

Sections of formalin-fixed and paraffin-embedded (FFPE) tissue 4 μm thick were stained with the Ventana ALK (D5F3) rabbit monoclonal primary antibody, together with the rabbit monoclonal negative control immunoglobulin, Optiview DAB IHC detection kit, and an Optiview amplification kit on a Ventana BenchMark ULTRA stainer (Ventana Medical Systems, USA). Immunoreactivity was evaluated as positive when the tumor (any percentage of positive tumor cells) showed intense granular cytoplasmic staining [22].

2.3. FISH

FISH was performed on FFPE tumor tissue Sections 3 μm thick using a break-apart probe specific for the ALK locus (Vysis ALK Break Apart FISH Probe Kit; Abbott Molecular, Des Plaines, IL, USA) according to the manufacturer's instructions. At least 100 representative tumor cells were counted, and the presence of ALK gene rearrangement was concluded if ≥15% of the tumor cells showed a split red and green signal and/or an isolated (single) red signal. Otherwise, the specimen was classified as ALK FISH-negative. As criteria for ALK copy number gain (CNG) have not been established, the following cutoff value was adapted from previous research [23]: gain (including both low and high genomic gain) was defined as a mean copy number of three to five fusion signals in ≥10% of analyzed cells. Quality control of the hybridized specimens was performed, and specimens with evidence of chromatin over-digestion or poor probe penetration were not acceptable and needed be retested after trouble-shooting the technical conditions. Tumor cells, the nuclei of which had one or more FISH signals of each color, were counted [22].

2.4. NGS

A 5-μm section on a hematoxylin-and-eosin-stained slide was reviewed by a pathologist to evaluate the tumor purity and encircle the tumor area. Genomic DNA was extracted from FFPE tumor tissues using TIANamp Genomic DNA Kit (Tiangen, Beijing, China). The quality of the DNA obtained was assessed by agarose gel electrophoresis, Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA), and Qubit 2.0 fluorometric quantification (Thermo Fisher Scientific, Waltham, MA, USA) to observe DNA concentration. Genomic DNA was randomly sheared into 150–300 bp segments by a Covaris S220 Focused-ultrasonicator (Covaris Inc, MA, USA). After end repair and dA-tailing, indexed adaptors were ligated to DNA fragments to perform pre-capture PCR. We adapted a custom-designed panel that covers ALK based on an Agilent liquid-phase hybrid capturing system for target enrichment. The manufacturers' recommendations were followed during the liquid-phase hybridization step. Following library enrichment, post-capture PCR was performed to yield final sequencing libraries. The fragment size of the prepared library was qualified using a 2100 Bioanalyzer (Agilent Technologies, Basel, Switzerland) and library concentration was quantified using qPCR NGS library quantification kit (Agilent Technologies, Basel, Switzerland). Multiplexed libraries were sequenced at the Novogene sequencing facility using a HiSeq platform (Illumina, San Diego, CA, USA).

RNA was extracted from FFPE tumor tissues using the RecoverAll™ Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA). The quality of the RNA obtained was assessed by qPCR and DNA concentration was assessed by agarose gel and Qubit 2.0 fluorometric quantification. RNA-based NGS was conducted using a custom-designed panel which detects ALK fusions. Libraries were prepared using a two-step PCR amplification method. The amplicon libraries were then sequenced with an Ion Torrent Systems Proton system (Thermo Fisher Scientific, Waltham, MA, USA), using a PI Chip, with barcoding performed using an Ion Xpress Barcode Adapter 1–96 kit. Data analysis was performed based on Torrent Suite™ Software v5.0 (Thermo Fisher Scientific, Waltham, MA, USA).

2.5. Treatment, response evaluation, and follow-up

The patient treatment information was obtained from electronic patient records (EPRs). In the patients who received first-line standard chemotherapy—either pemetrexed (500 mg/m^2 of body surface area; only case 24 was misdiagnosed as adenocarcinoma without an IHC test), docetaxel (75 mg/m^2), or gemcitabine (1250 mg/m^2 on days 1 and 8) plus cisplatin (75 mg/m^2)—the drugs were administered intravenously in 21-day cycles. The tumor response in this group was assessed every two cycles.

In the patients who received crizotinib, the dosage administered was 250 mg orally twice daily in 28-day cycles. The tumor response in this group was assessed after the first cycle of treatment and subsequently after every two cycles. Tumor responses were assessed using the RECIST (Response Evaluation Criteria in Solid Tumors), version 1.1 [24]. During follow-up, CT scans of the thorax and enhanced MRI of the brain were used to assess the response to standard chemotherapy and crizotinib. Responses to treatment were reported as complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD).

2.6. Statistical analysis

The association between ALK rearrangement status and clinicopathological data was assessed using SPSS software, version 17.0 (SPSS, Chicago, IL, USA), and the analysis was by the chi-square test.

Two-sided p value < 0.05 was considered statistically significant.

3. Results

3.1. Clinicopathological characteristics and ALK testing

To investigate whether IHC provides an effective approach to identifying LSCC patients who respond to ALK-targeted therapy, we reviewed 2403 consecutive cases of LSCC from three institutes in which all LSCC specimens were routinely examined with IHC. Overexpression of ALK protein occurred in a total of 37 patients (1.5%), as shown by IHC. The demographic characteristics of patients were detailed (see Supplemental material Table S1, which shows the characterization and ALK gene detection status of the 37 IHC-positive cases). Eight of the cases (21.6%) were female and 29 (78.4%) were male. The median age was 64 years (range 32–76). Twenty-five (67.6%) patients had a history of smoking. The pathological stagings were I, II, III and IV in seven (21.2%; IA2 two, IA3 three, IB two), nine (27.3%; IIA three, IIB six), 12 (36.4%; IIIA five, IIIB six, IIIC one) and five (15.1%; IVA five) patients, respectively. There were 18 surgically resected samples and 19 biopsies; 29 cases (78.4%) were recorded as focal positive and eight (21.6%) as diffuse positive.

Twenty-eight cases qualified for FISH, including four ALK-rearrangement-positive and 24 negative patients. The relationship between clinical parameter and ALK gene status by FISH is summarized in Supplemental Table S2. Among 28 patients, the median age was 64 (range 32–76) years. Five cases (17.9%) were female and 23 (82.1%) were male. Twenty-two cases (84.6%) had a history of smoking, four cases (15.4%) had never smoked, and in two cases smoking information was unavailable. The pathological stagings were classified as early stage (IA1–IIIA) in 17 cases (68.0%; IA2 two, IA3 two, IB one, IIA three, IIB six, IIIA three) and advanced stage (IIIB–IV) in eight cases (32.0%; IIIB four, IIIC one, IVA three). Three cases lacked sufficient clinical staging information. Four cases (14.3%) showed a diffuse staining pattern by IHC, while 24 cases (85.7%) showed focal expression.

In the group of patients with ALK gene rearrangement (four cases), the median age was 57 (range 42–73) years. Of these, three cases were female. Two cases had a history of smoking, two cases were at an advanced stage, and two cases showed diffuse ALK IHC expression.

In summary, ALK gene rearrangement was significantly more common in female patients (p = 0.011), while age, smoking status, clinical staging and ALK staining pattern showed no significant differences (p = 0.353, 0.099, 0.400 and 0.086, respectively) between the two groups (ALK-rearranged versus ALK non-rearranged by FISH) (Supplemental Table S2).

3.2. Low specificity of IHC for detection of ALK rearrangement

To evaluate the concordance between IHC and FISH, the 37 cases were subsequently tested by FISH for ALK rearrangement; however, nine cases failed the quality control due either to insufficient tumor cells or to no hybridization signals. Among the 28 IHC-positive cases with successful FISH testing, only four cases were positive for ALK rearrangement; the number of neoplastic nuclei harboring the ALK split pattern ranged from 20% to 33% (Table 1 and Fig. 1). Two cases (5 and 32) showed focal positivity with IHC, whereas the other two (14 and 24) showed diffuse positivity. The concordance rate of IHC compared with FISH in detecting ALK rearrangement was only 14.3% (4/28).

It was not feasible to perform FISH on all 2403 cases to evaluate the sensitivity of IHC in detecting ALK rearrangement. Alternatively, we addressed the above issue by randomly testing 40 IHC-negative cases with FISH. All 40 cases were FISH-negative, indicating good specificity of IHC compared with FISH.

3.3. Discrepancy revealed by next-generation sequencing

To further explore the low positive predictive value of IHC for ALK arrangement, we applied targeted DNA sequencing and RNA sequencing for 37 IHC-positive cases. However, only 13 samples passed quality control and gave NGS results for downstream analyses. Out of four FISH- and IHC-positive cases, only two cases (14 and 24, Fig. 1) showed *EML4-ALK* gene fusions—*E13: A20* and *E6: A20* respectively—in both DNA and RNA sequencing, consistent with IHC- and FISH-positive results. In the other two cases (5 and 32, Fig. 1) sequencing detected no ALK alteration. Further analyses revealed that case 5 showed *ARID1A (V5fs*106), PTEN (Q298X)* and *ERBB3 (P779 L)* gene point mutations; with *KLHL6* gene amplification, case 32 had *MET (M1L), TP53 (E310X), NOTCH1 (I1184fs*261)* and *PIK3CA (R108_E109insEEKILS)* gene point mutations, as well as *EGFR* and *CCND1* gene amplification. Among nine FISH-negative and IHC-positive cases, two cases (2 and 7) carried *ALK* point mutations *(K1525E and C928fs*11)*, while the remaining seven cases carried other aberrations such as *MYC* and *KRAS* amplifications, and mutations in *EGFR (I580 T), PIK3CA (E726 K, H1047R), MAPK (E33X, D398 A), ROS1 (L590 P, G747 V, T145 P and I537 M)* and *mTOR (E1799 K)* (see Supplemental Table S1).

3.4. High incidence of ALK copy number gain (CNG)

Among the 68 cases with FISH analysis—which consisted of 28 cases with ALK IHC-positive and 40 cases presenting as ALK IHC-negative—31 cases (45.6%) showed ALK CNG. Among 27 cases which were both ALK IHC- and ALK FISH-negative for rearrangement, CNG ranged from low to high (see Supplemental Figure S1). Three cases (22, 23 and 25) were ALK IHC-positive but ALK FISH-negative for rearrangement (see Supplemental Figure S2), and one case (case 32) was ALK IHC-positive and ALK FISH-positive for rearrangement (Fig. 1). The percentage of ALK CNG in IHC-negative cases was higher than that in IHC-positive cases (27/40, 67.5% versus 4/28, 14.3%).

3.5. Unsatisfactory clinical outcomes with crizotinib treatment in four ALK IHC positive LSCC patients

The clinical information on four patients who received crizotinib are summarized in Table 2. Case 2 showed focal positivity with IHC, was rearrangement-negative by FISH, and had an ALK point mutation *(K1525E)* by NGS. The patient showed no response to crizotinib and remained in SD for 3 months, then subsequently received therapy with PD1 immune checkpoint inhibitors which continues to the present time. Case 10 showed diffuse positivity with IHC, was rearrangement-negative by FISH, and had no NGS data because of lack of sufficient samples.

Table 1
Detailed information of the four ALK-rearranged lung squamous-cell carcinomas (LSCCs) detected by fluorescence in-situ hybridization (FISH).

Case	Age	Gender	SS	Stage	VIHC	FISH split signals	NGS DNA	NGS RNA
5	61	Male	SM	IA3	Moderately to strongly focal positive	20%	Without ALK associated gene	Without ALK-associated gene
14	42	Female	NS	IIIC	Strongly diffuse positive	32%	*EML4-ALK (E13:A20)*	*EML4-ALK (E13:A20)*
24	53	Female	NS	IIIA	Strongly diffuse positive	33%	*EML4-ALK (E6:A20)*	*EML4-ALK (E6:A20)*
32	73	Female	SM	IVA	Moderately to strongly focal positive	25%, with CNG	Without ALK-associated gene	Without ALK-associated gene

SS, smoking status; VIHC, Ventana immunohistochemistry; NGS, next-generation sequencing; NS, non-smoker; SM, smoker; CNG, copy number gain.

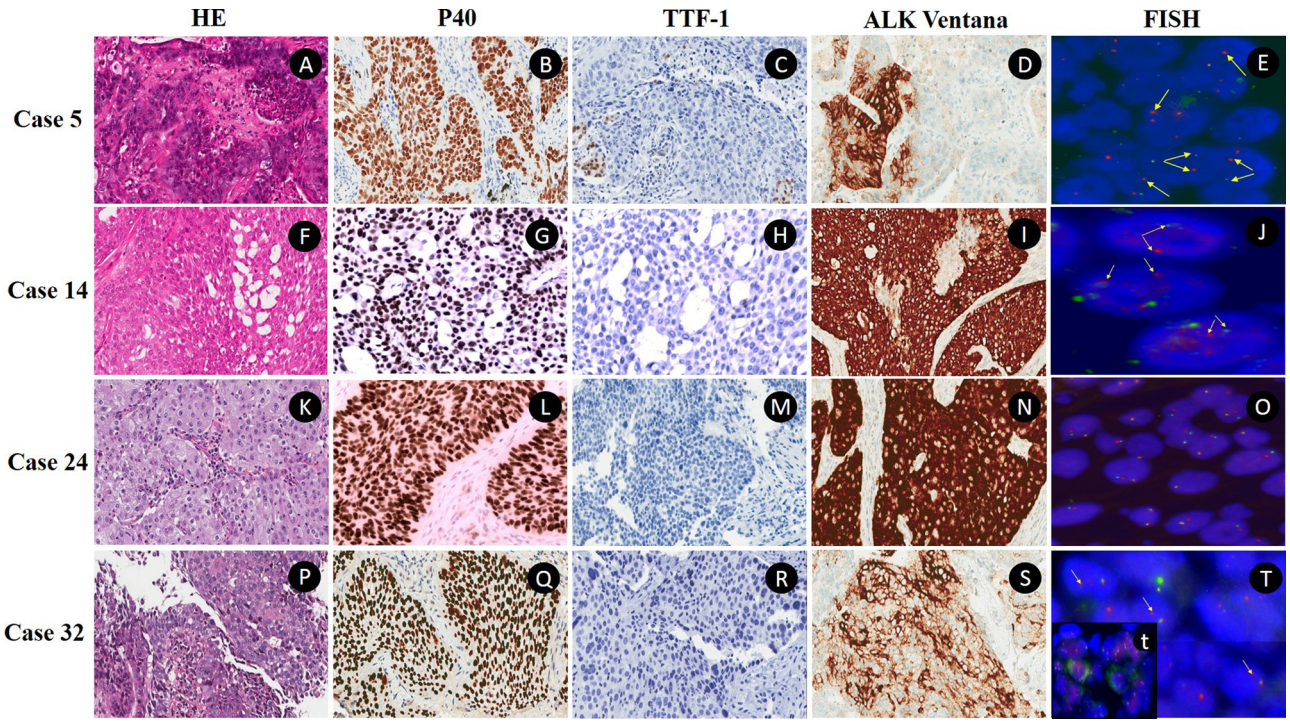

Fig. 1. Four ALK-rearranged lung squamous-cell carcinoma cases which were entirely identified by positive P40 expression (B, G, L, Q) and negative TTF-1 expression (C, H, M, R). Case 5 (A–E) showed moderate to strong focal expression of ALK protein by Ventana D5F3 ALK immunohistochemistry (IHC) (D). ALK gene was proved to be rearranged, and the percentage of split cells was evaluated as 20% (E). Case 14 (F–J) showed strong diffuse expression of ALK protein on IHC (I). Fluorescence in-situ hybridization (FISH) was positive for ALK gene rearrangement, and the percentage of split cells was evaluated as 32% (J). Case 24 (K–O) showed strongly diffuse expression of ALK protein on IHC (N), and the number of positive apart cells was evaluated as 33% by FISH (O). Case 32 (P–T) showed moderate to strong focal expression of ALK protein in the cytoplasm on IHC (S) and simultaneously showed ALK rearrangement (a single red signal) (T) and copy number gain (t), and the number of ALK signals was calculated as 25%. Magnification in all cases was 200 × . Yellow arrows indicate split signals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

The patient was diagnosed at an advanced stage, had PD upon crizotinib treatment for 2 months, and died of multiple organ failure. Case 24 (Fig. 2) showed diffuse positivity with IHC, ALK rearrangement by FISH, and *EML4-ALK* gene fusion *(E6:A20)* by NGS. The patient received crizotinib therapy for 9 months, and showed a moderate response to crizotinib therapy in the first 3 months; the clinical efficacy was evaluated as SD (reduced 20%). In the next 6 months the lesion remained stable, then the patient was enrolled in a new clinical trial and is still alive. Case 32 (Fig. 3) showed focal positivity with IHC, ALK rearrangement and ALK CNG by FISH, and no ALK gene alterations by NGS. The patient was diagnosed at an advanced stage, and was treated with crizotinib for a total of 10 months. In the first 8 months the focus showed no response to crizotinib and remained stable. However, in the next 2 months the patient rapidly exhibited pleural effusion and pulmonary consolidation, and finally after receiving crizotinib died in hospital emergency 10 months later; the clinical efficacy was evaluated as PD.

Table 2
Clinicopathological characteristics and treatment outcomes of ALK protein overexpression in lung squamous-cell carcinoma (LSCC) patients who received crizotinib therapy.

Case	Age	Sex	Stage	SS	FISH	NGS	Treatment	Time of TKI medication	Time to progression	Response to TKI	Clinical efficacy	Outcome
2	63	M	NA	SM	Negative	PM: *ALK (K1525E)*	Chemotherapy, crizotinib, immunotherapy	3 months	–	No	SD	Alive
10	32	F	IIIB	NS	Negative	Lack of samples	Chemotherapy, crizotinib	2 months	2 months	No	PD	Died
24	53	F	IIIA	NS	Positive (33%)	*EML4-ALK (E6:A20)*	Chemotherapy, operation, radiation, crizotinib	9 months	–	Yes[a]	SD	Alive
32	73	F	IVA	SM	Positive (25%), with CNG	PM: *MET (M1L)* and *PIK3CA (R108_E109insEEKILS)*	Chemotherapy, radiation, crizotinib	10 months	8 months	No	PD	Died

SS smoking status; FISH, fluorescence in-situ hybridization; NGS, next-generation sequencing; TKI, tyrosine kinase inhibitor; NS, non-smoker; SM, smoker; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; CNG, copy number gain; NA, not available; PM, point mutation.

[a] Indicated the response of the lesion of case 24 to crizotinib after treatment 3 months later (reduced about 20%); however, according to the criterion of RECIST (version 1.1), clinical efficacy was SD, then the focus remained stable for the next 6 months.

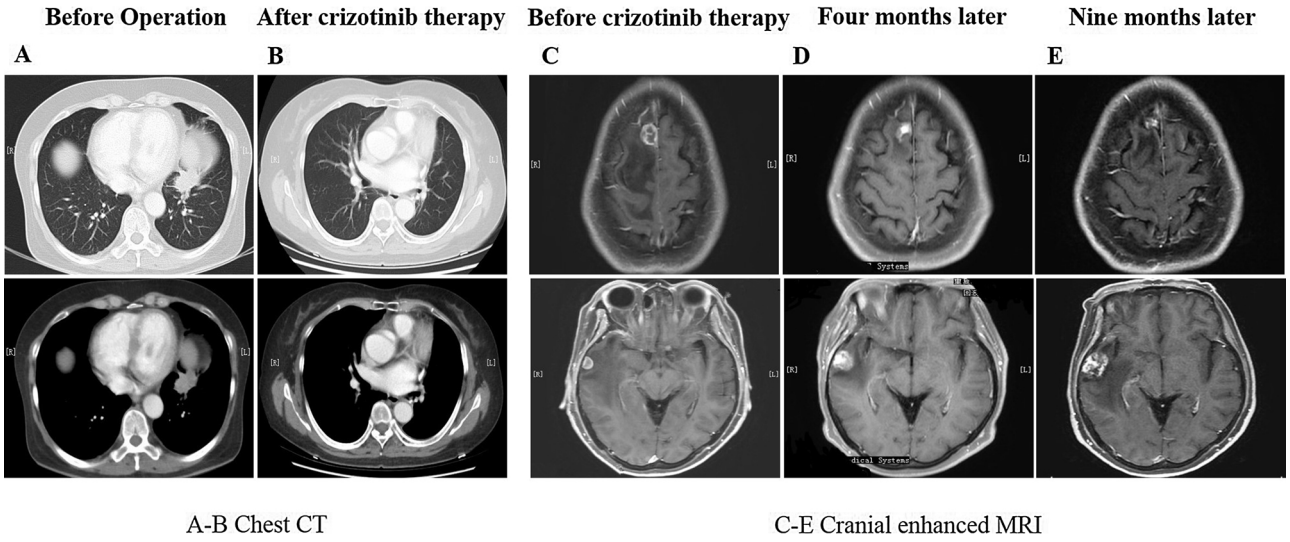

Fig. 2. Chest computed tomography (CT) scans for case 24. CT showed the response to first-line chemotherapy before surgical resection (A), then the patient underwent tumor resection in the left lower lobe and received postoperative adjuvant chemoradiotherapy and showed no tumor growth. Nine months ago, crizotinib was administrated, resulting in a stable response with no tumor growth up to the present time (B). Cranial enhanced magnetic resonance imaging (MRI) findings before and after crizotinib (4 months later and 9 months later). MRI before treatment revealed that (C) two metastatic loci were observed in the right frontal lobe and right temporal lobe, respectively. After treatment 4 months later (D) and 9 months later (E), the lesion of the frontal lobe remained stable while the lesion of the temporal lobe slightly increased.

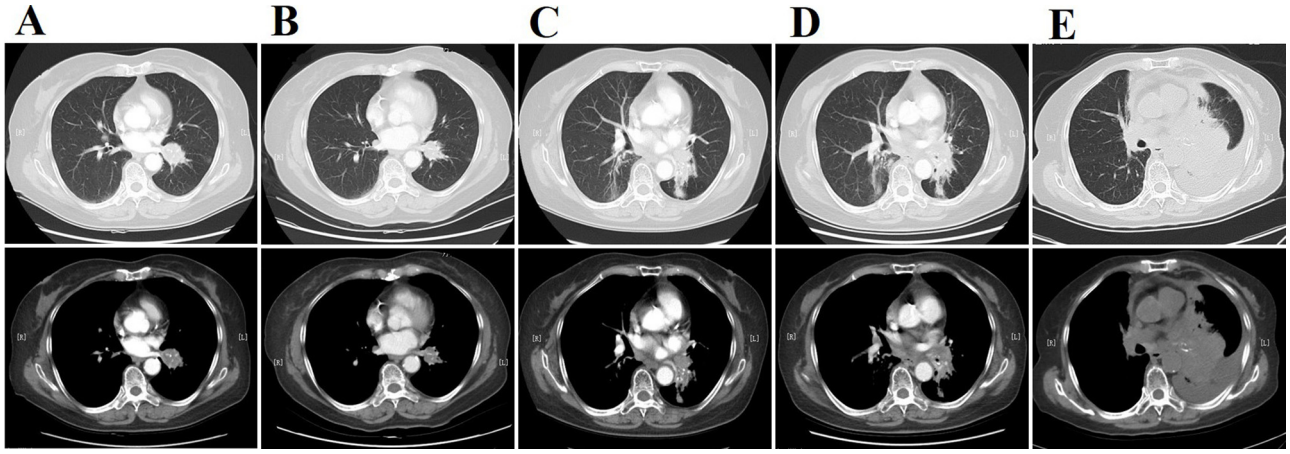

Fig. 3. Computed tomography (CT) scans for case 32. The chest CT showed changes in the primary lung carcinoma (A), after the first-line chemotherapy (B), a month later with crizotinib treatment (C), 4 months later with crizotinib treatment (D), and 8 months later with crizotinib treatment with enlargement of the pulmonary consolidation (E). The response to crizotinib was evaluated as progressive disease (PD).

4. Discussion

ALK rearrangement is one of the most important molecular alterations in NSCLC, particularly in LADC, and the incidence has been reported as ranging from 2% to 13% [2]. However, in previous studies in patients with LSCC ALK rearrangement was detected with a low frequency of 0–1.36% [7,12–15,23,25], while other investigators have reported a higher proportion of ALK rearrangement [26,27]. Our study—involving the screening of a large number of LSCC cases from multicenter institutions (three centers, 2403 cases) with IHC and further confirmation by FISH—revealed an ALK gene fusion rate of 0.17%. In addition, the cases with ALK protein expression detected by IHC were reconfirmed as LSCC using IHC markers. Thus, our study provides a realistic prevalence of the ALK rearrangement rate in LSCC, at least among a Chinese population. The higher proportion of ALK gene fusion in LSCC in previous publications could be attributed to the lack of IHC marker confirmation for the pathological diagnosis of LSCC, or it might include tumors with components of LADC.

The ALK rearrangement in LADC patients was significantly associated with younger patients, females, and non-smoking status [28]. As shown in Table 3 [15–21,29], ALK-rearranged LSCC tended to occur in patients < 60 years, those at an advanced stage, and Asian patients. In our study, the status of ALK-rearranged LSCC was associated exclusively with female patients, with no correlation with age, smoking status or clinical staging.

In patients with LADC, IHC with the D5F3 clone has been compared with FISH in many studies, and the use of D5F3 has shown high sensitivity and specificity (ranging from 83% to 100%) [3,30]. Therefore, because of the limitations of FISH (including low throughput technology and the need for specialized training to interpret results), IHC could be used as an acceptable alternative to FISH or as a screening diagnostic method for ALK gene abnormalities in LADC [7]. Moreover, the IHC ALK (D5F3) platform is now employed as a routine diagnostic approach in LADC for clinical targeted therapy [11]. However, studies on the correlation between ALK protein expression and ALK gene fusion in LSCC have seldom been reported. In our study, among 37 ALK IHC-positive cases, 28 cases were also analyzed by ALK FISH, and only four cases proved positive for ALK rearrangement. The concordance rate of

Table 3
Literature review of lung squamous-cell carcinomas (LSCCs) with ALK rearrangement to date.

Authors	Age	Gender	ALK detection	Smoking history	Clinical staging	Ethics	Clinical efficacy
Watanabe et al. [15] case 2	65	Female	IHC, FISH[a]	SM	IV	Japan	PD
Watanabe et al. [15] case 3	36	Male	IHC, FISH	SM	IIIB	Japan	NA
Watanabe et al. [15] case 4	62	Male	IHC, FISH[b]	SM	IV	Japan	PD
Mamesaya et al. [16]	52	Female	IHC, FISH	NS	IV	Japan	PR
Tamiya A et al. [17]	78	Male	IHC, FISH	SM	T3Nx	Japan	PD
Takanashi et al. [18]	60	Male	IHC, FISH	SM	T2aN0M0 (Ib)	Japan	NA
Zhang et al. [19]	55	Female	IHC	NS	T1aN1M1b(IV)	China	PR
Mikes et al. [20]	36	Male	IHC, FISH	NS	NA	Austria	PR
Wang et al. [21]	55	Female	IHC, FISH	NS	T4N3M1(IV)	China	PR
Vergne et al. [29]	58	Female	IHC, FISH	NS	IV	French	PR
Our series case 5	61	Male	IHC, FISH	SM	T1bNx	China	NA
Our series case 14	42	Female	IHC, FISH, NGS	NS	IIIC (T4N3M0)	China	NA
Our series case 24	53	Female	IHC, FISH, NGS	NS	IIIA (T2bN2M0)	China	SD
Our series case 32	73	Female	IHC, FISH	SM	IV (T1cN3M1)	China	PD

ALK, anaplastic lymphoma kinase; IHC, immunohistochemistry, FISH, fluorescence in situ hybridization; NGS, next generation sequencing; NS, non-smoker; SM, smoker; PD, progressive disease; PR, partial response; SD, stable disease; NA, not available.
[a] Case 2 was ALK FISH-positive/IHC-negative.
[b] Case 4 was ALK FISH-positive/IHC-negative.

ALK protein expression and ALK gene rearrangement was estimated as 14.3%, which was much lower than that in LADC. Therefore, ALK protein positivity by IHC is considered unreliable to guide TKI therapy for LSCCs and requires further confirmation by FISH. Reviewing previous articles, the explanation for this discordance between IHC and FISH includes the following points: (1) false-negative explanation of FISH results, especially for the results that are close to the cutoff value of 15% [31]; (2) amplification of the ALK gene (which might relate to ALK protein expression in some but not all cases), possibly resulting in 1+ or 2+ staining [23,32]; (3) false-positive interpretation of the IHC test results; and (4) an indeterminate mechanism [33,34]. Actually, it was one of our original motivations to explore the mechanism. However, due to insufficient samples or poor quality of DNA extraction, only 13 cases passed quality control, and we could not further explore the internal mechanism by NGS. Thus, we plan to collect more surgical ALK IHC-positive cases to explore the molecular alterations in the future.

Normal lung tissue does not express ALK protein; thus, theoretically speaking, any type of ALK gene alterations might result in ALK protein expression and ALK IHC positivity. ALK gene amplifications and CNGs in NSCLC are frequent. In a retrospective analysis of 69 LADCs, the frequency of ALK CNG was a little higher than that in LSCCs (65.2% versus 60.0%) [23]. Additionally, when compared to cases with ALK gene rearrangement, ALK CNG was significantly more common in ALK non-rearranged tumors [35,36], which suggests that ALK rearrangement occurs early in tumorigenesis, preceding chromosomal instability [35,37]. However, the copy number changes in ALK and their significance have been poorly characterized in NSCLC. In our study, the overall incidence rate of CNG was estimated as 45.6%, and ALK CNGs in IHC-negative cases were higher than those in IHC-positive cases, which is consistent with the results presented previously in the literature. Our observation suggests poor correlation between IHC and ALK CNG: in other words, increased CNG exhibited no association with ALK protein expression in LSCC [22].

In our study, two cases demonstrated EML4-ALK gene fusion on NGS, which was consistent with protein expression by IHC and rearrangement by FISH. Two other IHC-positive cases and ALK gene mutations were also observed, which indicated that ALK protein expression might also be associated with ALK gene mutation as well as gene fusion. However, two ALK-rearranged LSCC cases demonstrated by FISH showed no ALK mutation except for *MET, EGFR,* and *PIK3CA* alterations by NGS, suggesting that—although the technology was strictly quality controlled [38,39] to ensure accuracy in our study—NGS might still give false-negative results.

The results detailed above suggest that LSCCs, in addition to having a fusion rate lower than that in LADCs, may also have ALK gene CNG and ALK gene mutation. This study demonstrated that the ALK alternative in LSCC might to be more complicated than that in LADC. The relationship between ALK expression in LSCC and ALK gene status (such as rearrangement, CNG, mutation) and other genes from alternative pathways is unclear. Therefore, when discussing ALK status in NSCLC, separate descriptions for LADC and LSCC should be made.

Similarly, with ALK fusions, activating mutations in EGFR is also uncommon in LSCC. Whether EGFR-mutated LSCC patients also benefit more from the EGFR TKIs remains unclear. Recently Lu et al [40] reported a pooled analysis for the efficacy of EGFR TKIs in targeted therapy of LSCC patients with EGFR mutation, and revealed that EGFR TKIs had only a modest efficacy for LSCC patients with EGFR mutation compared with EGFR wild-type patients, and might be a selective option for patients with EGFR mutation. Shukuya et al also reported that EGFR-TKIs are less effective in non-adenocarcinoma NSCLC with EGFR mutation than in LADC harboring EGFR mutation [41]. The mechanism of the lower response rates to EGFR-TKI treatment in LSCC patients with EGFR mutation compared to LADC patients harboring EGFR mutation is unclear.

Similarly to the uncertain clinical efficacy of EGFR-TKI in LSCC, it is also debatable whether LSCC patients with ALK rearrangement could benefit from ALK inhibitors. Crizotinib yielded very high response rates when used in advanced patients with ALK-positive NSCLC [1]. Although ALK-rearranged LSCC has been reported in case reports and remarkable responses have been observed by some authors [18–21,26,29], there were also reports of poor effectiveness [15,17]. In our study, four cases of LSCCs with ALK-positive expression on IHC had undergone targeted therapy with crizotinib. Two of them had been confirmed by FISH to have ALK gene fusion. The only case whose ALK rearrangement was confirmed by both FISH and NGS remained stable for 9 months, which is evaluated as SD. The other one with ALK rearrangement and ALK CNG was clinically evaluated as PD. A recent study reported that the high ALK gene CNG is not a driver genetic event in lung cancer tumorigenesis, but it might represent a marker of chromosome instability and correlate with an aggressive metastatic behavior [42]. Moreover, the increase in ALK CNG emerges as a mechanism of resistance to crizotinib treatment [43]. Therefore, the ineffectiveness of ALK TKI in patients with ALK CNG might be attributed to other factors. The other two cases showed merely ALK-protein-positive expression, with no evidence of ALK gene fusion; thus it is difficult to further interpret the molecular mechanism of the lower responses (SD and PD for each) to crizotinib therapy when compared to LADC. A recent study has reported that the RAS-RAF-MEK-ERK signal pathway controls the ALK inhibitor response in ALK-positive lung cancer and is critical for ALK inhibitor resistance [44]. Another probable explanation

might be the complex genomic alterations in LSCC compared with LADC, which was identified by the Cancer Genome Atlas Research Network [45,46].

In general, the treatment effectiveness of two cases with ALK gene fusion in this group was evaluated as SD or PD, so the therapeutic results were not as good as in LADC. Despite the poor outcomes, ALK inhibitor crizotinib remains a feasible treatment option in ALK-rearranged LSCCs except for standard chemotherapy or chemotherapy/radiotherapy. However, what would be the most cost-effective approach and the testing algorithm to identify those ALK-positive LSCC patients with such low prevalence? Upon systematic review, a reasonable strategy might be to test patients younger than 60 years, females with advanced-stage disease, and particularly Asian patients.

There were some limitations to this study. The testing results of ALK-positive cases by IHC screening was incomplete due to lack of residual tissue for further FISH and NGS detection. In addition, the patients with ALK FISH results had only limited data on ALK TKI therapy. Further studies on the effectiveness of ALK inhibitor therapy in ALK IHC- and/or FISH-positive LSCC patients is warranted.

5. Conclusion

In LSCCs, the frequency of ALK protein expression determined by IHC was 1.5%, and ALK gene rearrangement by FISH was 0.17%; the correlation between IHC and FISH was 14.3%, which is far less than that in LADC. In addition, our study showed that ALK protein expression might not only correlate with ALK gene rearrangement but also with ALK gene mutation, while ALK CNG occured in 45.6% of cases but showed no association with ALK protein expression. Compared with LADC, screening for ALK-positive expression with IHC is not considered as a reliable method for LSCCs. Moreover, patients with ALK-rearranged LSCCs appeared to have poorer response rate to crizotinib than those with LADC.

Conflict of interest

None.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2019.02.015.

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