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Lung Cancer

Discordance between Fluorescence In Situ Hybridization and Immunohistochemistry Analysis of Anaplastic Lymphoma Kinase Rearrangement in Indian Patients with Non-Small Cell Lung Cancer

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Abstract

Aims This study aims to evaluate the incidence of anaplastic lymphoma kinase (ALK) mutation in nonsmall cell lung cancer (NSCLC) incorporating fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC) methods and to look for any discordance.

Methods We evaluated 101 samples obtained from an enriched cohort of NSCLCs patients from the Army Hospital Research and Referral, New Delhi, India, between November 2016 and November 2018. IHC was performed using the highly-sensitive D5F3 rabbit monoclonal primary antibody. FISH was performed with dual-color, break-apart probe (ZytoLight SPEC) on formalin-fixed, and paraffin-embedded tissue. Discordance between IHC and FISH for ALK rearrangements was evaluated. Pearson correlation coefficient (*r*) was performed to identify any association of ALK presence (by IHC and FISH) with smoking brain metastasis, programmed death-ligand (PD-L1) expression, pleural effusion, and histopathological subtype.

Results A total of 7.92% (8/101) cases tested by IHC and 9.9% (10/101) cases tested by FISH were positive for ALK rearrangement. Of 93 ALK IHC-negative cases, 4 were ALK FISH-positive, whereas of 91 ALK FISH-negative cases, 4 were ALK IHC-positive cases. The correlation analysis demonstrated no or very weak correlation in ALK mutations by IHC or FISH with smoking, brain metastasis, PD-L1 expression, pleural effusion, and histopathological examination, except a weak positive correlation ($r = 0.33$) observed between brain metastasis and ALK rearrangement identified by FISH.

Conclusions Our study demonstrated a somewhat similar incidence of ALK FISH-positive cases and ALK IHC-positive cases, though the incidence was numerically higher for ALK-FISH method.

Keywords
- anaplastic lymphoma kinase rearrangement
- fluorescence in situ hybridization
- immunohistochemistry
- non-small cell lung cancer

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Introduction

The detection of anaplastic lymphoma kinase (ALK) genetic alterations and epidermal growth factor receptor (*EGFR*) activating mutations has facilitated the use of targeted therapies for nonsmall cell lung cancer (NSCLC).[1-4]

Several methods, including karyotyping, fluorescence in situ hybridization (FISH), immunohistochemistry (IHC), real-time polymerase chain reaction (RT-PCR), and next-generation sequencing, have been used for ALK testing,[5-9] but the preferred method is debatable. We compared IHC (D5F3 clone) versus FISH for ALK testing in Indian patients with NSCLC. Correlations between ALK presence and smoking, brain metastasis, programmed death-ligand (PD-L1) expression, pleural effusion, and histopathological examination were also evaluated.

Methods

Study Design

The data of 101 proven adult (>18 years) cases of NSCLC (adenocarcinoma) registered at Malignant Diseases Treatment Centre, Army Hospital Research and Referral, New Delhi, India, between November 2016 and November 2018 are presented. This was an observational study, and all patients who gave consent for the study were included. The patient's samples were simultaneously sent for EGFR by PCR, ALK, and ROS1 by IHC and FISH methods. A detailed patient history including the age, sex, smoking status, symptoms on presentation, duration of symptoms, cancer stage, presence of brain metastasis, and histopathological evaluation (well-differentiated, poorly differentiated moderately differentiated, or adenosquamous status) was recorded.

Fluorescence In Situ Hybridization for Anaplastic Lymphoma Kinase Rearrangement

Fish was performed using a dual-color break-apart probe (ZytoLight SPEC) in formalin-fixed, paraffin-embedded (FFPE) tissue. All general reagents used were sourced from Sigma unless otherwise specified. Paraffin blocks of 4 to 5 µm thick section were taken on positively charged glass slides. The slides were incubated at 80°C (±2°C) for 45 minutes and thereafter dewaxed in two changes of xylene (Merck) for 20 minutes each, followed by two immersions of 5 minutes each in 100% ethanol (Merck). The slides were air-dried and then sequentially immersed in 0.2 N hydrochloric acid for 20 minutes, in 2× SSC for 5 minutes at room temperature followed by 1M sodium isothiocyanate for 35 to 40 minutes at 80°C followed by immediate immersion in 2× SSC for 5 minutes at room temperature. The slides were then incubated for 10 to 25 minutes at 37°C in 1% pepsin solution. The slides were then dehydrated using gradations of alcohol (70%, 85%, and absolute ethanol for 1 minute each) and then air dried. Five microliters of the ready to use mixture of ALK Probe (ZytoVision Cat # Z-2124–200) were added to the center of a 12 mm × 12 mm coverslip (Bluestar). The coverslip was lifted gently by touching it to the marked hybridization area of the glass slide (the probe hybridization needed to be performed in dark). The hybridization area was sealed using rubber cement and was allowed to dry. The slides were placed in a humidified ThermoBrite hybridization chamber (Abbott) and incubated for 80°C for 10 minutes, followed by 37°C for 14 to 16 hours.

After incubation, the slides were removed from the humidified chamber and were processed for washing to remove the unbound probe. The slides were incubated in Solution C (1 mL of 20× SSC and 150 µL NP-40 added to 49 mL MQ) at 72°C (±1°C) for 2 minutes and were immediately followed by incubation in Solution D (5 mL of 20× SSC and 50 µL NP-40 added to 45 mL MQ) for 2 minutes at room temperature.

The slides were removed from Solution D, air-dried, and then mounted in a solution of 5 µL DAPI II (Vysis, Abbott). The slides were visualized on a BX-61 Olympus fluorescence microscope equipped with Cytovision software and the nuclei were scored as per guidelines. One hundred nuclei were counted (50 each by 2 readers). Negative and positive calibrator control slides from Abbott were used to establish a measurement of uncertainty (MU) for ALK FISH testing; MU was established to be 7%. A sample was considered FISH negative if <5 cells out of 50 were positive, while it was considered positive if >25 cells out of 50 were positive. A sample was considered equivocal if 5 to 25 cells out of 50 were found positive. In such cases, a second reader also evaluated the slide. In the final analysis, if <15% cells out of 100 were positive, then the sample was considered "negative," while if >15% had translocations, it was considered "positive."

Immunohistochemistry for Anaplastic Lymphoma Kinase Rearrangement

IHC test was performed using anti-ALK (D5F3) rabbit monoclonal primary antibody along with Optiview DAB IHC detection and Optiview amplification kits by automated method on Ventana Benchmark XT. All reagents used were sourced from Ventana. Paraffin blocks of 2 to 3 µm thick section were taken on poly-L-lysine coated slides. The sections were immersed in skimmed milk for 10 to 15 minutes and subsequently dried with tissue paper. The slides were incubated on heating pads for deparaffinization using a reagent cell conditioning solution (Antigen Unmasking). Preprimary peroxidase inhibitor was applied. ALK (D5F3) antibody was applied and the slides were incubated at 37°C temperature for 16 minutes followed by OV HQ UNIV Linker (12 minutes incubation), OV HRP multimer (12 minutes incubation), OV AMP H202 and OV Amplifier (8 minutes incubation), OV AMP multimer (8 minutes incubation), hematoxylin (counterstain) (8 minutes incubation), and bluing reagent (post counterstain) (4 minutes incubation). The slides were dehydrated, cleared, and mounted with DPX. Liquid coverslip was applied at each step as per the Ventana protocol. Scoring criteria for the determination of ALK status in NSCLC were considered positive if there was a presence of strong granular cytoplasmic staining in tumor cells (any percentage of positive tumor cells). Known staining elements were excluded, including light cytoplasmic stippling in alveolar macrophages, cells of neural origin (nerve and ganglion cells), glandular epithelial staining, and cells within lymphocytic infiltrate. Some

background staining also may be observed within normal mucosa in NSCLC (including mucin) and in necrotic tumor areas, which were also excluded from the clinical evaluation.

Epidermal Growth Factor Receptor Mutation Analysis

The *EGFR* assay was based on PCR and gene sequencing analysis. The analytical sensitivity of the test allows the detection of the mutation when the mutant clone comprises at least 18 to 20% of the total genomic deoxyribonucleic acid (DNA), including differentiation and apoptosis. The tumor samples were fixed under appropriate conditions (10% neutral buffered formalin for 6–48 hours; 12 hours for small biopsies) to ensure the preservation of amplifiable quality DNA. The mutations were screened in exons 18 to 21 of the *EGFR* gene in the FFPE tissue blocks. This test was developed and its performance was evaluated at Oncquest Laboratories Ltd., New Delhi, India.

Fluorescence In Situ Hybridization Test Validation

The FISH test was validated using "Intertechnology Orthogonal" validation technique. Thirty FFPE samples of NSCLC that had previously tested "Negative" for EGFR mutation were used for the validation study as per the protocol described in methods. Results of FISH testing using ALK Probe (ZytoVision Cat # Z-2124–200) were compared with IHC testing using D5F3 antibody clone on the Ventana platform. A concordance of 88% was observed between the two techniques. In addition, the laboratory routinely participates in CAP Proficiency Testing surveys and National EQAS.

Study Assessments

The study end points included the evaluation of discordance between IHC and FISH for ALK rearrangements. The association of ALK presence (by IHC and FISH) with smoking, brain metastasis, PD-L1 expression, pleural effusion, and histopathological examination was also evaluated.

Statistical Analysis

The data collected were entered into Microsoft Excel 2010 (Microsoft Corporation, United States) and analyzed with SAS Version 9.4 (SAS Institute Inc., United States). The demographic variables were expressed in numbers and percentages. The relationship between ALK presence (by IHC and FISH) and smoking, brain metastasis, PD-L1 expression, pleural effusion, and histopathological examination was established using Pearson correlation coefficient (r) method.

Results

A total of 101 proven cases with NSCLC (adenocarcinoma) were evaluated. The mean age of the patients was 57.9 (range: 27–83) years, and majority were male (75.25%). Majority (86.14%) of the patients had Stage IV cancer and presented with hemoptysis and cough (23.76%) followed by chest pain and dyspnea (22.77%) symptoms. The mean (standard deviation) duration of presentation of symptoms was 3.7 (2.65) months (range: 1–12 months). Histopathological evaluation revealed poorly differentiated NSCLC in 35.64% of patients followed by moderately differentiated in 28.71% patients. The previous treatment included concurrent chemoradiotherapy in Stage III NSCLC patients presenting with metastatic disease. Stage IV NSCLC patients had received first- or second-line chemotherapy and were reported for further management.

►**Table 1** presents the baseline demographic and characteristics of the patients.

The IHC testing revealed a positive ALK rearrangement in 7.92% (8/101) cases, whereas it was positive in 9.9% (10/101) cases tested by FISH testing. Furthermore, of 93 ALK IHC-negative cases, 4 cases were ALK FISH-positive cases, whereas of 91 ALK FISH-negative cases, 4 were ALK IHC-positive cases (►**Table 2**).

Of eight ALK IHC-positive cases, only one (12.5%) patient was a smoker, brain metastasis was present in three (37.5%) patients, PD-L1 expression in four (50%) patients, and pleural effusion in three (37.5%) patients. PD-L1 expression in these four patients was 1, 5, 15, and 50%, respectively.

Similarly, of ten ALK FISH-positive cases, four (40%) patients were smokers, brain metastasis was present in five (50%) patients, PD-L1 expression in three (30%) patients, and pleural effusion in five (50%) patients. PD-L1 expression in these three patients was 1, 10, and 15%, respectively.

Pearson correlation coefficient evaluated for ALK rearrangement by IHC showed no or very weak correlation with confounding factors including smoking, brain metastasis, PD-L1 expression, and pleural effusion ($r < 0.3$ for all) (►**Table 3**). Similarly, there was no or very weak correlation established for ALK rearrangement by FISH with smoking, PD-L1 expression, and pleural effusion ($r < 0.3$ for all), but a weak positive correlation was observed for the presence of brain metastasis ($r = 0.32765$).

Table 1 Patient disposition and baseline characteristics ($n = 101$)

Parameters	Value
Age (y), mean ± SD (range)	57.9 ± 11.26 (27–83)
Sex, *n* (%)	
Men	76 (75.25)
Women	25 (24.75)
Smoking present, *n* (%)	61 (60.40)
Cancer stage, *n* (%)	
II	1 (0.99)
III	13 (12.87)
IV	87 (86.14)
Brain metastasis present, *n* (%)	15 (14.85)
Histopathology evaluation, *n* (%)	
Well-differentiated	14 (13.86)
Moderately differentiated	29 (28.71)
Poorly differentiated	36 (35.64)
Adenosquamous disease	22 (21.78)
Previous treatment received, *n* (%)	13 (12.87)

Abbreviations: *n* = number of patients; SD = standard deviation.

Table 2 Results for anaplastic lymphoma kinase rearrangement in non-small cell lung cancer by fluorescence in situ hybridization and IHC methods

Parameters	*n*/*n* (%)
EGFR mutation present	11/101 (10.89)
ALK rearrangement by IHC	
Positive	8/101 (7.92)
Brain metastasis present in ALK IHC-positive	3/8 (37.50)
PD-L1 expression present in ALK IHC-positive	4/8 (50)
Pleural effusion present in ALK IHC-positive	3/8 (37.50)
HPE in patients with positive ALK rearrangement by IHC	
Well differentiated	2/8 (25)
Moderately differentiated	2/8 (25)
Poorly differentiated	2/8 (25)
Adenosquamous disease	2/8 (25)
ALK rearrangement by FISH	
Positive	10/101 (9.90)
Brain metastasis present in ALK FISH-positive	5/10 (50)
PD-L1 expression present in ALK FISH-positive	3/10 (30)
Pleural effusion present in ALK FISH-positive	5/10 (50)
HPE in patients with positive ALK rearrangement by FISH	
Well-differentiated	1/10 (10)
Moderately differentiated	4/10 (40)
Poorly differentiated	2/10 (20)
Adenosquamous disease	3/10 (30)

Abbreviations: ALK, anaplastic lymphoma kinase; EGFR, epidermal growth factor receptor; FISH, fluorescence in situ hybridization; HPE, histopathology evaluation; IHC, immunohistochemistry; PD-L1, programmed death-ligand.

Of 101 patients, 11 (10.89%) were positive for *EGFR* mutations. The mutation test covers exon 18 to 21 of the *EGFR* gene. The results showed that 27.3% (3/11) of these patients were Dell9 positive. Of 11 patients positive for *EGFR* mutation, one patient showed ALK rearrangement by IHC method and none of the patients by FISH method. Del 19 was not seen in this patient. This patient had Stage IV moderately differentiated NSCLC with brain metastasis; PD-L1 expression and pleural effusion were not observed.

Discussion

The current study determined the presence of *EGFR* mutations, ALK rearrangement, and evaluated FISH and IHC methods for ALK rearrangement testing in Indian patients with NSCLC.

Table 3 Correlation analysis

Parameter	Pearson correlation coefficient	
	ALK rearrangement by IHC	ALK rearrangement by FISH
Smoking	–0.287	–0.138
Brain metastasis	0.187	0.328
PD-L1 expression	0.101	–0.026
Pleural effusion	0.095	0.204
Well-differentiated HPE	0.095	–0.037
Moderately differentiated HPE	–0.024	0.083
Poorly differentiated HPE	–0.065	–0.108
Adenosquamous HPE	0.023	0.066

Abbreviations: ALK, anaplastic lymphoma kinase; FISH, fluorescence in situ hybridization; HPE, histopathology evaluation; IHC, immunohistochemistry; PD-L1, programmed death-ligand.

Previous studies have concluded the noncoexistence of *EGFR* and ALK mutations.[10] Gainor et al demonstrated that ALK rearrangement and *EGFR* mutations are mutually exclusive. An analysis of 1683 NSCLC patients demonstrated that none of the patients showed overlapping ALK and *EGFR* mutations.[11] However, a previous report by Yang et al has indicated a rare co-expression of EGFR and ALK rearrangement in lung cancer patients.[12] Our study demonstrated that ALK rearrangement (identified by IHC) was present in one patient in whom *EGFR* mutation was also present.

The ALK rearrangement testing for lung cancer has become a standard practice according to the College of American Pathologist and the European Society of Medical Oncologists Guideline.[3] The FISH method is the standard choice as it has a relatively simpler technique than other molecular techniques and is able to directly visualize the translocation in the ALK gene with appropriate probes. However, the interpretation of ALK by FISH is difficult in NSCLC patients as ALK positivity in NSCLC is an intrachromosomal rearrangement, which may lead to a close separation of the break-apart probes.[13] Hence, several other methods are also used like IHC.

A meta-analysis by Dearden et al demonstrated the incidence of ALK mutation in 2 to 7% of NSCLC cases.[14] The incidence of ALK mutation is reported lower in Indian studies, from 2 to 3%.[15,16] In our study, the ALK rearrangement was evaluated using IHC (D5F3 rabbit monoclonal primary antibody) and FISH (dual color break-apart probe ZytoLight SPEC) methods. The study reported ALK mutation in 7.92% of cases evaluated by IHC (8/101) and 9.9% of cases evaluated by FISH (10/101). The relatively high incidence of ALK rearrangement in Indian patients observed in our study could be attributed to the fact that majority of the patients had brain metastasis, and a higher incidence of ALK positivity is reported in

patients of NSCLC with brain metastasis.[17,18] Furthermore, other studies have reported outcomes of patients from a relatively confined target population whereas in our study patients were included from a multicenter referral hospital with patients being treated from a wide geographical area, may explain the possible higher incidence pertaining to the heterogeneity among different regions.

A recent study by Scattone et al reported 13.6% ALK FISH-positive cases and 6.3% ALK IHC-positive cases from a cohort of 95 NSCLC patients.[1] Desai et al reported ALK gene rearrangement in 2.7% of patients through FISH method in Indian patients with NSCLC in a single-center retrospective study (n = 224 patients).[16] A multicenter study that evaluated ALK mutations through FISH method showed ALK rearrangement in 3% of 500 NSCLC adenocarcinoma patients in India.[15] Chatterjee et al reported an ALK mutation incidence of 2.19% by FISH method versus 5.02% by IHC method in 379 Indian NSCLC patients.[19]

In the current study, of ten cases that were ALK FISH-positive, four were also ALK IHC-positive cases. Similarly, of eight ALK IHC-positive cases, four were also ALK FISH-positive. In a study by To et al, all 20 FISH-positive cases showed positive ALK protein expression as determined by IHC.[20] Another study by Moatter et al demonstrated that four of the seven ALK FISH-positive cases were also ALK IHC-positive.[2]

The incidence of brain metastasis is reported up to 40% in NSCLC patients.[21] Furthermore, NSCLC patients with ALK-positive rearrangement are at a higher risk of developing brain metastases.[22] Rangachari et al reported that brain metastasis was present in 23.8% ALK-FISH positive NSCLC patients.[17] Of 947 ALK-positive NSCLC patients from three databases from US routine clinical practice, 28% of patients had brain metastases.[18] Our study demonstrated the presence of brain metastasis in 37.5% ALK IHC-positive cases and 50% ALK FISH-positive cases. Furthermore, correlation analysis showed a weak positive correlation (r = 0.328) with ALK FISH-positive cases and brain metastasis, whereas there was no or very weak positive correlation (r = 0.187) with ALK IHC-positive cases and brain metastasis. A positive correlation between ALK and higher PD-L1 levels are reported in preclinical studies, although the underlying mechanisms are not established.[23] In our study, PD-L1 expression was present in 50% ALK IHC-positive cases and 30% ALK FISH-positive cases. However, no or very weak correlation was established for PD-L1 expression and ALK rearrangement by FISH or IHC in our study. Similarly, a meta-analysis of 26 studies (n = 7541) demonstrated no significant correlation between ALK status and PD-L1 positivity in NSCLC patients.[23]

The incidence of ALK mutation in smoker patients with lung cancer is generally low (2%).[24] Similarly, in our study, of 61 smoker patients, only 4 had ALK-positive mutations. Our study also evaluated the correlation and showed no or very weak negative correlation among smoking and ALK IHC (r =–0.287) or ALK FISH (r =–0.138).

Malignant pleural effusion is reported in 15% of patients with lung cancer during initial workup. In a study by Carter et al ALK gene rearrangement was reported in 3.7% (1/27 ALK FISH) of patients.[25] Our study showed pleural effusion in 37.5% ALK IHC-positive patients and 50% ALK FISH positive patients, thought the correlation analysis demonstrated no or very weak correlation. Wang et al investigated the correlation between ALK mutation and clinicopathological features in patients diagnosed with lung adenocarcinoma and demonstrated no correlation of ALK mutation with gender, smoking history, pleural effusion, lymphatic metastasis, or clinical staging, similar to that reported in our study.[26]

Our study evaluated the *EGFR* mutation using a technique based on PCR and gene sequencing analysis in the exons 18 to 21 of the *EGFR* gene from the FFPE tissue samples. The diagnosis of *EGFR* mutation allows targeted tyrosine kinase inhibitor treatments, which may be considered the best choice of treatment for NSCLC.[27] *EGFR* mutation is reported in 10 to 30% of NSCLC cases as per previous reports.[28] Our study demonstrated the presence of *EGFR* mutations in 10.89% of the patients, similar to the reported evidence. A previous large cohort study from India (n = 907) demonstrated 23% EGFR mutation in NSCLC patients.[28] The exon 19 deletion (19del) and point mutation on exon 21 (L858R) are common *EGFR* mutations.[29] In our study, L858R was the most common mutation reported in seven patients followed by Del 19 in three patients, and one patient had unidentified mutation.

Conclusion

In conclusion, we report that the incidence of ALK rearrangement was somewhat similar for FISH and IHC methods, though the incidence was numerically greater for FISH method. These results need to be confirmed in a large population to draw any conclusion on the preferred method for ALK rearrangement testing in NSCLC.

Disclaimer

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patients have given their consent for their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflict of Interest

There are no conflicts of interest.

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