Original Article

Molecular epidemiological study of microtubule-associated protein-like 4-anaplastic lymphoma kinase fusion gene using immunohistochemistry as a cost effective alternative to fluorescence *in situ* hybridization for Indian patients with adenocarcinoma lung

ABSTRACT

Background: With fluorescence *in situ* hybridization (FISH) as the main-stay for the detection of anaplastic lymphoma kinase (ALK) rearrangements, the ALK Break Apart FISH Probe Kit has become a Food and Drug Association-approved companion diagnostic for targeted therapy with the ALK inhibitor crizotinib in lung cancers. The objective of this molecular epidemiological study was to estimate the prevalence of microtubule-associated protein-like 4-ALK (EML4-ALK) fusion gene using immunohistochemistry (IHC) as a cost effective alternative to FISH for Indian patients with nonsmall-cell cancer (NSCC)-adenocarcinoma, favor adenocarcinoma lung and NSCC- Not otherwise specified (NOS).

Materials and Methods: Patients with NSCC-adenocarcinoma, favor adenocarcinoma lung, and nonsmall cell lung cancer-NOS histology were considered for this study. Permission was obtained from the Ethics Committee before the start of the study. Clinical characteristics and treatment details were collected from the patient's medical records. IHC analysis was performed using a Ventana automated immunostainer (Benchmark XT). Detection was performed using OptiView DAB Detection and Amplification Kit.

Results: A total of 200 NSCC-adenocarcinoma, favour adenocarcinoma and NSCC-NOS patients were included in the study. There were 122 (61%) men and 78 (39%) women with a median age of 57 years. Of the 200 patients, 43 (21.5%) were nonsmokers and 175 (87.5%) had Stage-IV disease at the time of initial diagnosis. 48 (24%) cases were positive for epidermal growth factor receptor mutations, whereas EML4-ALK fusion gene was present in 27 (13.5%) patients. 25 of the 27 patients with ALK positivity received crizotinib therapy.

Conclusions: The incidence of EML4-ALK gene fusions (13.5%) in this Indian population is four-fold high than the previous reported incidences and supports the claim of several recent studies that a relatively new ALK clone, 5A4, and D5F3 from Leica/Novocastra and cell signaling technology/Ventana, respectively can accurately identify ALK rearranged lung adenocarcinoma. The inclusion of IHC for the detection of EML4-ALK gene fusions as a low cost alternative seems justified in low resource setting.

Keywords: Anaplastic lymphoma kinase, crizotinib, nonsmall cell lung cancer, Ventana

INTRODUCTION

Despite significant advancement in the detection and treatment of lung cancer, it still remains a leading cause of cancer-related death worldwide with an overall 5-year survival rate of just 15%.^[1] Efforts are underway to develop effective targeted therapy strategies in order to improve the survival of patients with advanced nonsmall-cell cancer (NSCC).

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Studies have shown that around 64% of NSCC patients harbor at least one activated pathway with mutations in either Kirsten RAS (KRAS) gene or epidermal growth factor receptor (EGFR) gene or the fusion gene of echinoderm microtubule-associated protein-like 4 (EML4) and the intracellular domain of anaplastic lymphoma kinase (ALK), named as EML4-ALK.^[2-7]

Crizotinib, an oral ATP-competitive tyrosine kinase inhibitors (TKIs) of ALK and c-MET, has shown a good response rate of 57% and progression free survival (PFS) of 72% in advanced NSCC, especially in the tumors harboring ALK rearrangements that are negative for EGFR mutation as well as amplification of MET.^[8] In an open label Phase 3 trial, crizotinib has demonstrated a median progression free survival and response rate of 7.7 months and 65% as compared to 3 months and 20%, respectively, in the chemotherapy group ($P < 0.001$).^[9] A very high 1 and 2 years overall survival of 77% and 64%, respectively has been reported with crizotinib therapy in patients with advanced NSCC.^[10] The presence of EGFR and EML4-ALK mutations in NSCC patients of Indian ethnicity has been reported in the range of 23%–44% and 2.7%–3%, respectively.^[11-15]

Although, the ALK Break Apart FISH Probe Kit (Abbott Molecular, Des Plaines, IL, USA) has become an Food and Drug Association (FDA)-approved companion diagnostic for targeted therapy with crizotinib in lung cancers, the assay can lead to false negatives and false positives along with a very high inter-reader variability.^[16] Furthermore, due to the requirement of specialized fluorescence microscopy equipment, fluorescence *in situ* hybridization (FISH) technique remains an expensive and time-consuming preposition. Immunohistochemistry (IHC) for ALK protein expression in nonsmall cell lung cancer (NSCLC) has been introduced to overcome the limitations of FISH technique. Several recent studies have demonstrated that staining of sections on Ventana immunostainer (Benchmark XT) using an antibody D5F3 and labeling as well as signal amplification kit OptiView by Ventana Roche have a high sensitivity and specificity when compared to FISH.^[17-19] Likewise, the 5A4 antibody demonstrate ALK1 protein overexpression has also been found to be satisfactory reports a sensitivity and specificity of 82%–100% compared to the gold standard of FISH.^[20-22] However; due to binary nature of interpretation and complete standardization of reagents, controls, and staining protocol Ventana IHC platform for detecting ALK (D5F3) expression has been approved by United States-FDA in the year 2015. We undertook this molecular epidemiological study to estimate the prevalence of EML4-ALK fusion gene using IHC as a cost effective alternative to FISH for Indian patients with adenocarcinoma lung.

MATERIALS AND METHODS

Patients with NSCC-adenocarcinoma, NSCC favor adenocarcinoma and NSCLC-NOS histology, whose tumors had been tested for EML4-ALK fusion gene using IHC were considered for this study. Permission was obtained from the Ethics Committee before the start of the study. Clinical characteristics and treatment details were collected from the patient's medical records. IHC was assayed on 4- μ m neutral buffered formalin fixed; paraffin-embedded tumor tissues using a primary rabbit monoclonal ALK antibody (mAb) clone D5F3 obtained from Ventana USA. IHC staining was performed using a Ventana Benchmark XT immunostainer (Ventana Medical Systems, Illkirch Graffenstaden, France). The slides were dried for 1 h at 60°C, deparaffinized for 4 min at 75°C using EZ Prep followed by incubation at a dilution of 1:50 for 1 h at 37°C with the primary mAb. Detection was performed using a multimer-technology system with the OptiView Universal DAB Detection Kit from the same vendor.

Statistical analysis was performed using Pearson Chi-square or Fisher's exact test, whichever was appropriate for categorical variables. Logistic regression was performed to compare the study groups. A two-sided $P < 0.05$ was considered statistically significant. Binary logistic regression with single independent variable was performed and therefore, statistical correction has not been applied to the P values. Statistical analysis was performed using SAS version 9.3 (SAS Institute Inc., Cary NC, USA).

RESULTS

A total of 200 NSCC adenocarcinoma patients were included in the study. There were 122 (61%) men and 78 (39%) women with a median age of 57 years. Of the 200 patients, 43 (21.5%) were nonsmokers and 175 (87.5%) had Stage-IV disease at the time of initial diagnosis. Baseline patient characteristics are presented in Table 1.

The result of molecular testing is shown in Table 2. 48 (24%) cases were positive for EGFR mutations whereas EML4-ALK fusion gene was present in 27 (13.5%) patients. 25 out of the 27 patients with ALK positivity received crizotinib therapy.

The association of each of the individual factor with regards to EGFR and EML4-ALK fusion gene mutation is shown in Table 3. While gender and cigarette smoking were significantly associated with EGFR mutation ($P < 0.05$) the EML4-ALK fusion gene mutation showed significant association with gender, cigarette smoking, and performance status ($P < 0.05$).

Table 1: Summary of patient demographic and tumour characteristics (n=200)

Characteristics	n (%)
Median age (years; range)	57 (22-84)
Gender (%)	
Male	122 (61)
Female	78 (39)
Smoking (%)	
Smokers	68 (34)
Never smokers	43 (21.5)
Unknown	89 (44.5)
Grade (%)	
Well differentiated	4 (2)
Moderately differentiated	13 (6.5)
Poorly differentiated	51 (25.5)
Unknown	132 (66)
Stage (%)	
I	-
II	-
III	3 (1.5)
IV	175 (87.5)
Unknown	22 (11)
Morphology	
Papillary	15
Lepidic	13
Solid	10
Acinar	9
Acinar, solid	4
Solid, lepidic	4
Acinar, papillary	3
Papillary, lepidic	2
Papillary, solid	2
Acinar, lepidic	1
Acinar, papillary, solid	1
Papillary, solid, lepidic	1
Unknown	135
IHC	
p-63	63
TTF, CK5/CK7	47
TTF	45
CK5/CK7	19
p-63, TTF	18
p-63, TTF, CK5/CK7	12
p-63, CK5/CK7	4

IHC - Immunohistochemistry; TTF - Thyroid transcription factor; CK - Cytokeratin

Table 2: Results of molecular testing (n=200)

Characteristics	n (%)
EGFR mutations	
Positive	48 (24)
Wild type	152 (76)
EML4-ALK fusion gene	
Positive	27 (13.5)
Wild type	173 (86.5)

EGFR - Epidermal growth factor receptor; EML4 - Echinoderm microtubule-associated protein-like 4; ALK - Anaplastic lymphoma kinase

Table 4 presents the distribution of EGFR and EML4-ALK gene mutations across the study population.

DISCUSSION

The molecular profiling of the tumor using companion diagnostics followed by subsequent treatment with the relevant targeted therapy has improved the overall survival as well as treatment outcomes in NSCLC. Data from recent studies indicates that the most common mutations in NSCLC are KRAS gene (24%), EGFR gene (13%–22%) and ALK gene rearrangements (5%–6%).^[6,7] The use of EGFR TKI for patients with EGFR activating mutations and an oral ATP-competitive TKI of ALK and c-MET, has shown a good response rate in advanced NSCLC. Ever since its approval in the year 2011, crizotinib, has unfolded a new era of personalized treatment in NSCLC tumors harboring ALK rearrangements.

The incidence of EGFR mutation in NSCC has been reported in the range of 13% for Caucasian population and 47% for Asian population.^[23] Various epidemiological studies of NSCC from India have reported the incidence of EGFR mutation in the range of 23.2%–51.8% with a dominance of female gender.^[11-15] The incidence of EGFR mutations (24%) in our study is lower than the one reported for Asian patients (47%) but much higher than that for Caucasian patients (13%). In the present study, a higher EGFR mutation rate was observed in females than males (12.5% vs. 11.5%, $P = 0.033$) and the results were found to be statistically significant. This observation is consistent with the previously published studies that have shown the female gender dominance in the EGFR mutation.^[2,13,24,25] The result of our study demonstrates a positive correlation of EGFR mutation in the never smoker group as compared to smokers (7.5% vs. 4.5%, $P = 0.029$).

The incidence of ALK gene rearrangement in NSCLC has been reported in the range of 3% to 13% for Caucasian and East Asian population using the FISH technique.^[4,26] Two studies from India have reported an incidence of ALK gene rearrangement in NSCLC as 2.7% and 3%, respectively, using the FISH technique whereas there is no published report on the incidence using the IHC assay.^[14,15] Several recent studies have demonstrated a high sensitivity and specificity of Ventana IHC platform for detecting ALK (D5F3) expression using OptiView labeling kit as compared to FISH or other IHC assays.^[17-19] Further, patients who are tested FISH-negative and IHC-positive creates diagnostic and therapeutic issues for clinicians in making a right treatment decision. A study by Sun *et al.* reports a dramatic response to crizotinib therapy in ALK-positive NSCLC patients who are tested FISH negative but IHC-positive.^[27] The incidence of ALK gene rearrangement (13.5%) in our study is much higher than

the one reported for Caucasian patients (3%) as well as the previously reported incidence for Indian population using FISH. In the present study, a higher ALK gene rearrangement rate was observed in females than males (8.0% vs. 5.5%, $P = 0.020$) and the results were found to be statistically significant. The higher positivity as established by IHC can be due to technical difficulties in FISH interpretation and

loss of genetic material between 5' portion of ALK gene and 3' portion of EML4 gene during inversion bringing back the signals close together and seemingly fused. The result of our study demonstrates a positive correlation of ALK gene rearrangement in the never smoker group as compared to smokers (3.0% vs. 2.0%, $P = 0.047$). Furthermore, there was a positive correlation of ALK gene rearrangement in stage IV patients (13.0%, $P = 0.012$).

Table 3: Association of each of the individual factor vis-à-vis epidermal growth factor receptor and echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase fusion gene mutations

Variable	EGFR mutations		EML4-ALK fusion gene mutations	
	χ^2	P	χ^2	P
Gender	4.5443	0.0330*	5.3852	0.0203*
Age	0.6808	0.7115	3.2135	0.2005
Cigarette smoking	7.0826	0.0290*	6.0983	0.0474*
Tobacco chewing	3.3058	0.0690	0.1328	1.0000
Alcohol intake	3.3670	0.1401	0.0262	1.0000
Histology	3.3655	0.3386	1.7945	0.6161
Stage of disease	3.2712	0.3517	0.5220	0.9140
PS	7.7636	0.1006	12.8401	0.0121*
IHC	9.8735	0.6271	10.8825	0.6207
Morphology	12.1345	0.5955	13.2901	0.5038

*Significant value. IHC - Immunohistochemistry; PS - Performance status; EGFR - Epidermal growth factor receptor; EML4 - Echinoderm microtubule-associated protein-like 4; ALK - Anaplastic lymphoma kinase

CONCLUSION

Our study reports the first Indian data on EML4 ALK fusion gene mutations using IHC as a cost effective alternative to FISH for Indian patients with adenocarcinoma lung. The incidence of EML4-ALK gene fusions (13.5%) in this Indian population is four-fold high than the previous reported incidences and supports the claim of several recent studies that a relatively new ALK clone, 5A4 and D5F3 from Leica/Novocastra and cell signaling technology/Ventana, respectively can accurately identify ALK rearranged lung adenocarcinoma as compared to FISH. The inclusion of IHC for the detection of EML4-ALK gene fusions as a low cost alternative seems warranted.

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Nil.

Table 4: Distribution of epidermal growth factor receptor and echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase K gene mutations

Variable	EGFR			EML4-ALK fusion gene		
	Wild type (%)	Mutated (%)	P	Wild type (%)	Mutated (%) Y	P
Gender						
Female	53 (26.5)	25 (12.5)	0.0330*	62 (31.0)	16 (8.0)	0.0203*
Male	99 (49.5)	23 (11.5)		111 (55.5)	11 (5.5)	
Age						
20-40	11 (5.5)	4 (2.0)	0.7115	11 (5.5)	4 (2.0)	0.2005
40-60	77 (38.5)	27 (13.5)		89 (44.5)	15 (7.5)	
> 60	64 (32.0)	17 (8.5)		73 (36.5)	8 (4.0)	
Smoking history						
Never smokers	30 (15.0)	15 (7.5)	0.0290*	39 (19.5)	6 (3.0)	0.0474*
Smoker	59 (29.5)	9 (4.5)		64 (32.0)	4 (2.0)	
Unknown	63 (31.5)	24 (12.0)		70 (35.0)	17 (8.5)	
Stage						
I	0	0	0.3517	0	0	0.9140
II	0	0		0	0	
III	2 (1.0)	1 (0.5)		3 (1.5)	0	
IV	130 (65.0)	45 (22.5)		149 (74.5)	26 (13.0)	
Unknown	20 (10.0)	2 (1.0)		21 (10.5)	1 (0.5)	
PS						
1	26 (13.0)	14 (7.0)	0.1006	34 (17.0)	6 (3.0)	0.0121*
2	19 (9.5)	3 (1.5)		14 (7.0)	8 (4.0)	
3	8 (4.0)	0		7 (3.5)	1 (0.5)	
4	4 (2.0)	0		3 (1.5)	1 (0.5)	
Unknown	95 (47.5)	31 (15.5)		115 (57.5)	11 (5.5)	

*Significant value. EGFR - Epidermal growth factor receptor; EML4 - Echinoderm microtubule-associated protein-like 4; ALK - Anaplastic lymphoma kinase; PS - Performance status

Conflicts of interest

There are no conflicts of interest.

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