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Epidermal growth factor receptor (*EGFR*), *KRAS*, and *BRAF* mutations in lung adenocarcinomas: A study from India

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A B S T R A C T

Mitogen-Activated Protein (MAP) Kinase pathway involves several oncogenic genes which can serve as potential targets for therapy. Therefore, aim of the present study is to analyze mutations in the MAP Kinase pathway in pulmonary adenocarcinoma (ADCA) of Indian patients along with clinico-pathologic correlation and determination of the survival status in patients receiving therapy. Blocks and slides of 125 pulmonary ADCA of last 5 years were retrieved. Histo-morphology and tumor content were determined. *EGFR, KRAS, BRAF* and *MEK1* genes were analyzed using Sanger sequencing and Real-time polymerase chain reaction (PCR). Clinico-pathologic correlation and survival analysis were performed. Fifty-eight (46.4%) patients harbored genetic mutations of which 49 had single somatic mutations, 5 had multiple exonic and 4 showed coexisting *EGFR* and *KRAS* mutations. *EGFR* mutations were seen in 24.8%, *KRAS* in 19.2% and *BRAF* (non-V600E) in 2.4% cases. There was no difference in progression-free survival of wild- type/single mutations when compared with multiple/ coexisting mutations ($P = 0.09$). However, the P value may indicate borderline correlation. To conclude, *EGFR* and *KRAS* mutations may coexist in the same patient in lung ADCA. Multiple exonic mutations of *KRAS* gene formed substantial percentage of our cohort, requiring further exploration. Lung ADCA harbouring *BRAF* mutations are commonly non-V600E. Testing of all major genetic driver mutations of lung ADCA irrespective of histology and other demographic characteristics is necessary.

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Introduction

Constitutive activation of signaling pathways is a common occurrence in human cancers and is often associated with molecular alteration of the key components of the signaling cascade.[1,2] One such intracellular signaling pathway playing an important role in lung cancer progression and development is the mitogen-activated protein (MAP) kinase pathway. Aberrantly activated MAP kinase pathway has been associated with tumor development and chemotherapy resistance.[1,3] This pathway is evolutionarily conserved and can be activated by several factors such as mitogens, growth factors, hormones, chemokines, and cytokines.[1,3,4] It consists of a cascade of signaling molecules which are sequentially activated.[1,2,4]

Epidermal growth factor receptors (*EGFR*) are tyrosine kinases receptors which are overexpressed or mutated in various malignancies including lung cancer.[5] Binding of epidermal growth factor (EGF) to *EGFR* activates *RAS* which is then followed by sequential activation of *RAF* and finally mitogen-activated protein kinase kinase *(MEK)*.[1,2,4]

EGFR-MAP kinase pathway has been studied intensively and inhibitors against *EGFR, RAS, RAF*, and *MEK* have been developed that target different components of this signaling cascade.[1-3] Moreover, it is a commonly known fact that *EGFR* and other driver mutations of lung cancer are mutually exclusive. However, with the advancement in molecular technology, multiple and coexisting *EGFR* mutations have been detected, without much exhaustive studies on their clinical significance.[6]

Moreover, complete and concurrent analysis of *EGFR* and MAP kinase signaling genes has not been explored widely in nonsmall cell lung carcinoma (NSCLC) from India; except for 1 study where only Kristen Rat Sarcoma Viral oncogene *(KRAS)* and v-raf murine sarcoma viral oncogene homolog B *(BRAF)* genes were analyzed.[7]

Therefore, the aim of the present study is to analyze the mutations in the *EGFR*-MAP kinase pathway in pulmonary adenocarcinoma (ADCA) in Indian patients, along with its clinicopathologic correlation and determination of the survival status in patients receiving targeted therapy. We also intended to compare our findings with the existing world literature.

Material and methods

Sample collection and diagnosis

One hundred and twenty-five cases of pulmonary ADCA diagnosed between January 2012 and December 2017, where adequate material was available, were retrieved from the archives of the Department of Pathology after approval from institute's ethics committee. The hematoxylin and eosin (H&E) stained slides were analyzed and histologic type of the tumor was determined according to World Health Organization 2015 classification of tumors of the lung, pleura, thymus, and heart.[8] Immunohistochemistry for thyroid transcription factor-1 was done in morphologically undifferentiated cases for definite characterization. Cases showing histomorphologically low-grade patterns namely acinar, lepidic, and papillary were grouped together as good prognostic histology (Group 1). Those having high-grade patterns such as solid, micropapillary, or sarcomatoid were grouped together as poor prognostic histology (Group 2) and invasive mucinous carcinomas were kept as a separate group (Group 3).[8] Similarly, tumors in stage I and II which are resectable were grouped together and stages III and IV tumors which are unresectable were kept in a separate group. Blocks showing more than 50% tumor component in their respective sections were used for DNA extraction and mutation analysis. All cases of small cell carcinoma, lung metastases, and those showing predominant necrosis were excluded from this study. Treatment and follow-up details were retrieved from case record files. Patients were managed in a multidisciplinary clinic as per stage, Eastern Cooperative Oncology Group (ECOG) performance status, and molecular profile which were available at the time of treatment decision making. Treatment response were assessed radiologically and labeled according to RECIST v 1.1.

Progression-free survival was calculated from date of diagnosis till date of disease progression or death.

DNA isolation and quantification

DNA extraction was performed using 50-µm-thick sections of formalin-fixed paraffin embedded tissue samples. Formalin-fixed paraffin embedded DNA tissue extraction kit (cat. No. A2352, Promega) was used for DNA extraction. The isolated DNA was assessed both qualitatively and quantitatively by spectrophotometry (Nanodrop, Biodrop Resolution, Cambridge, UK).

Real-time polymerase chain reaction (PCR)

The *EGFR* RGQ PCR Kit (cat no. 870111, Therascreen, Qiagen Ltd, Manchester, UK) was used for detecting the presence of 29 *EGFR* mutations spanning exons 18-21. *KRAS* PCR Kit (cat no. 870001 Therascreen, Qiagen Ltd, Manchester, UK) was used for detecting 7 *KRAS* mutations in exon 2 (codons 12 and 13). The analysis was performed according to the manufacturers' instructions.

Sanger sequencing

PCR was carried out to amplify exons using G2 colorless master mix (cat no. M7422 Promega) on ABI Palm thermal cycler (Applied Biosystem, California), using exon-specific primers. 3 µL of the purified PCR product was used. The sequencing was done using both forward and reverse primers for greater accuracy and the results were analyzed using SeqMan II software (DNASTAR).

Statistical analysis

Data analysis was done using statistical software Stata 14.0 (StataCorp LLC, Texas). Categorical data were expressed as frequency and percentage and quantitative data was expressed as mean $\pm$ standard deviation and median (minimum and maximum). Chi-square test and/or Fisher-exact test, independent *t* test, and rank-sum were used to check the statistical significance of the data. Survival analysis (Kaplan-Meier) was used to check the time to event (recurrence and/or metastasis) relationship. A P value <0.05 was considered significant.

Results

Patient characteristics

Of the 125 samples included in the study, 40 were resections and 85 were small biopsies. There was male predominance with a male: female ratio of 2.6:1. Median age was 58 years (26-85 years). Eighty-six (68.8%) patients were smokers, 72% of which were males. Ninety-five (76%) cases were in advanced clinical stage (stage III/IV). Approximately 90% patients were clinically well preserved with ECOG performance status between 0 and 2. Treatment history and follow-up status were known in 80 cases. Of these 80, 59 (73.7%) patients

Table 1
Clinicopathological parameters and mutation profile of pulmonary adenocarcinoma patients.

	Total N = 125	*EGFR* +ve N = 31	*KRAS* +ve N = 24	*BRAF* +ve N = 3	Coexisting mutations N = 4	Multiple mutations N = 5
Median age (years)	58	60	65	55	63	60
Male: Female	2.6:1	2.1:1	3:1	3:0	3:1	1.5:1
Smoker	86(68.8%)	19(61.2%)	18(75%)	2 (66.6%)	2(50%)	3(60%)
Non smoker	39(31.2%)	12(38.7%)	6 (25%)	1(33.3%)	2(50%)	2(40%)
Resection	40(32.0%)	11(35.4%)	8(33.3%)	1(33.3%)	2(50%)	0
Biopsy	85(68.0%)	20(64.5%)	16(66.6%)	2(66.6%)	2(50%)	5(100%)
Stage1 and 2	30(24.0%)	4(12.9%)	7(29.1%)	1(33.3%)	1(25%)	0
Stage 3 and 4	95(76.0%)	27(87.0%)	17(70.8%)	2(66.6%)	3(75%)	5(100%)
ECOG 0-2	112(89.6%)	26(83.8%)	23(95.8%)	3(100%)	4(100%)	5(100%)
ECOG 3-4	13(10.4%)	5(16.1%)	1 (4.1%)	0	0	0
Histology patterns						
Group 1 low grade	74 (59.2%)	24 (77.4%)	14 (58.3%)	1(33.3%)	3(75%)	5(100%)
Group 2 high grade	43 (34.4%)	7 (22.5%)	8(33.3%)	1(33.3%)	1(25%)	0
Group 3 mucinous	8 (6.4%)	0	2(8.3%)	1(33.3%)	0	0
Available treatment details						
	N = 80	N = 21	N = 16	N = 1	N = 3	N = 4
Chemo	59 (73.7%)	10 (47.6%)	14 (87.5%)	1 (100%)	2 (66.6%)	4 (100%)
TKI	21 (26.2%)	11 (52.3%)	2 (12.5%)	0	1 (33.3%)	0
Both	10 (12.5%)	7 (33.3%)	8 (50%)	0	1 (33.3%)	0
Treatment response						
	N = 61	N = 18	N = 14	N = 1	N = 3	N = 3
Group 1 (Stable/progressive)	44 (72.1%)	15 (83.3%)	8 (57.1%)	-	2 (66.6%)	1(33.3%)
Group 2 (Partial/Complete)	17 (27.8%)	3 (16.6%)	6 (42.8%)	1 (100%)	1(33.3%)	2 (66.6%)
Median PFS (in months)	8.3	8.1	8.3	–	7.3	

Abbreviations: Chemo, chemotherapy; ECOG, Eastern Cooperative Oncology Group; PFS, progression-free survival; +ve, positive; TKI, tyrosine kinase inhibitors.

received chemotherapy as first-line treatment and 21 (26.2%) were subjected to tyrosine kinase inhibitors (TKIs) as the therapy of choice. Demographic characteristics have been shown in Table 1.

Histopathologic examination

The most common histologic pattern of ADCA was acinar (78/125; 62.4%) followed by solid pattern (37/125; 29.6%). Around 10% (12/125) had lepidic morphology and 8.8% (11/125) revealed papillary architecture. Mucinous ADCA formed 6.4% (8/125) of study population; micropapillary and sarcomatoid patterns were seen in 1.6% (2/125) cases each. Ten cases showed undifferentiated morphology all of which were positive for thyroid transcription factor-1. Twenty percent (25/125) tumors showed mixed morphology with varying combinations of acinar, lepidic, solid, papillary and micropapillary patterns. Cases with low-risk patterns were put together in Group 1 (74/125; 59.2%) (Fig 1a–c) and those with even minor components of high-risk patterns were segregated in Group 2 (43/125; 34.4%) (Fig 1d and e). The mucinous pattern was included in Group 3 (8/125; 6.4%) (Fig 1f) (Table 1).

All histopathologic groups irrespective of the histology had significantly more number of patients in the higher stage ($P = 0.003$) (Fisher's exact test). Correlation of smoking status with histologic subtype also showed statistical significance with smokers being predominant in Groups

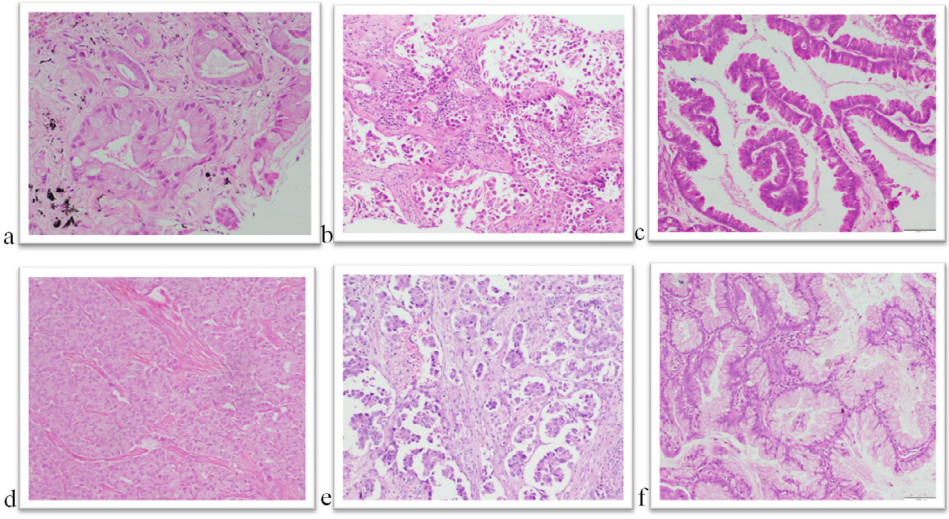

Fig. 1. Histomorphologic variants of pulmonary adenocarcinoma. (a) Acinar (Hematoxylin and Eosin (H&E) X 200). (b) Lepidic (H&E X 40). (c) Papillary (H&E X100). (d) Solid (H&E X100). (e) Micropapillary (H&EX100). (f) Mucinous (H&E X100).

1 (48/74; 64.8%) and 2 (36/43; 83.7%), whereas nonsmokers were predominant in Group 3 (6/8;75%) ($P = 0.002$) (Fisher's exact test).

Mutation distributions

Fifty-eight of 125 (46.4%) showed genetic mutations. Forty-nine of these had single mutations and 9 patients exhibited multiple mutations (which included multiple exonic mutations of the same gene or presence of multiple gene mutations in the same case). Overall, there were 24.8% (31/125) patients with *EGFR* mutation, 19.2% (24/125) with *KRAS* mutation and 2.4% (3/125) with *BRAF* mutation. The demographic parameters of individual genes have been shown in Table 1.

EGFR mutation

EGFR positivity had statistically significant correlation with histology, where the predominant pattern was acinar (group 1) and none were mucinous ($P = 0.036$) (Fisher's exact test). The most commonly detected mutations were exon 19 deletions (20/31) (Fig 2a and b) followed by exon 21 point mutations (5/31). The mutations of exon 21 were L858R (4/5 cases) and L861Q (1/5 case). Exon 20 insertion and T790M mutations were detected in 2 cases each. Exon 18 mutation (G719X) was found positive in 1 case. *EGFR* dual positivity was seen in 1 case (exon 19 deletion and exon 21 L858R point mutation) (Fig 2c) and had Group 1 histology.

KRAS mutation

KRAS positivity showed no significant correlation with histologic groups. *KRAS* mutation was seen mainly in codons 12 and 13 of exon 2. The most commonly detected mutations were G12A (6/24) (Fig 2d and e) and G12C (5/24), followed by G12V (4/24) and G12D (3/24). G12S and G13D mutations were detected in 1 sample each respectively. *KRAS* dual mutations were seen in 4 cases and revealed combinations of G12A and G12V in 3 cases and G12V and G12C co-positivity in 1 case (Fig 2f), all bearing Group 1 histology.

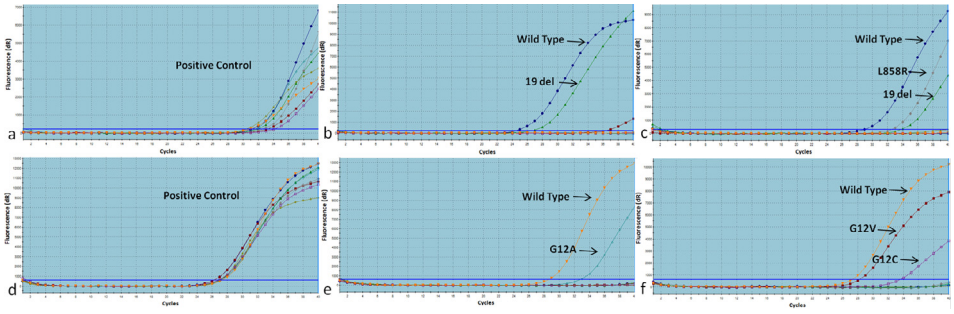

Fig. 2. Real-time PCR analysis of *EGFR* and *KRAS* mutations in pulmonary adenocarcinoma. (a) *EGFR* control assay. (b) *EGFR* exon 19 deletion. (c) *EGFR* exon 19 deletion and exon 21 L858R dual mutation. (d) *KRAS* control assay. (e) *KRAS* exon 2 G12A point mutation. (f) *KRAS* exon 2 G12V and G12C dual mutation.

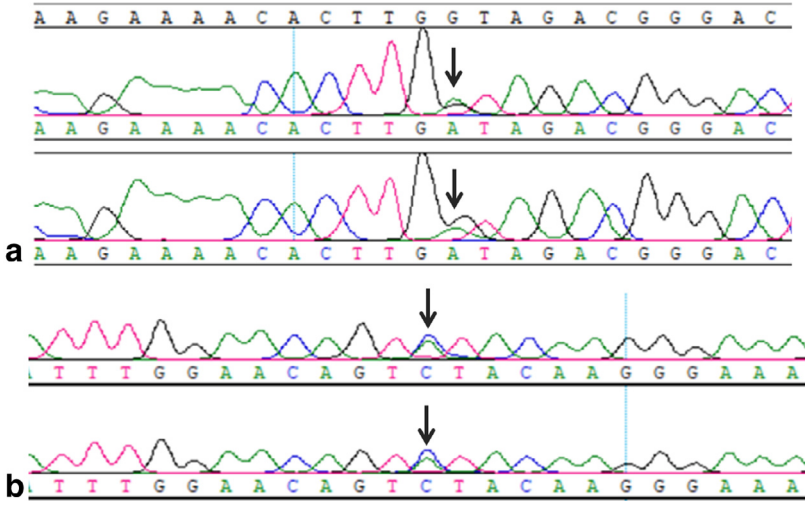

Fig. 3. Sanger sequencing analysis of *BRAF* mutation and polymorphism in pulmonary adenocarcinoma. (a) *BRAF* exon 11(G442D) point mutation. (b) *BRAF* polymorphism.

Coexistence of *EGFR* and *KRAS* mutations in the same patients were seen in 4 of 125 cases (3.2%). The combinations noticed were as follow:

a) *EGFR* Exon 18 G719X *KRAS* Exon 2 G12D
b) *EGFR* Exon 19 Del *KRAS* Exon 2 G12A
c) *EGFR* Exon 19 Del *KRAS* Exon 2 G12V
d) *EGFR* Exon 21 L858R *KRAS* Exon 2 G12C

Out of these 4 positive cases, 2 cases were reconfirmed by Sanger sequencing. Histologically, 3 of them showed acinar pattern (Group 1) and 1 was solid ADCA.

BRAF mutation

BRAF mutations consisted of point mutations in exon 11(G442D) (Fig 3a) in 1 case and exon 15 (L597V) in 2 cases. *BRAF* positivity had no histologic predominance as 1 case each fell into the 3 histologic groups. In addition, *BRAF* polymorphism was seen in 14.4 % cases (18/125) (Fig 3b). One case showed both *BRAF* polymorphism as well as L597V point mutation.

MEK1 mutation

None of the cases exhibited *MEK1* (exon1) mutation.

Follow-up

Out of 80 patients whose treatment details were available, 19 patients were lost to follow-up. To evaluate treatment response, we divided patients into 2 groups; response group 1 included cases with either stable disease or progressive disease (44/61; 72.1%) and response group 2 consisted of patients with either partial or complete response (17/61, 27.8%). We had a median follow-up of 5.5 months. The Kaplan-Meir curve analysis and log rank test did not show any significant difference in the median progression-free survival (PFS) of wild type or mutated patients ($P = 0.09$). We tried correlating progression with the different therapeutic regimens (TKI vs chemotherapy). However, due to limited number of patients with available follow-up in each subgroup, no reproducible statistics could be performed.

Due to small sample size and even smaller subgroup of patients in each mutation type, the power of this study is less than 80%.

Discussion

In NSCLC, the incidence of *EGFR* mutations varies considerably across different regions of the world.[9,10] Various studies from India have shown regional diversity with its frequency ranging from 23% to 51.8%.[11,12] One of these studies has compared the frequency of *EGFR* mutations in the population from North and South India and have found lower incidence of the mutation in people from North (33%) than Southern part of India (65%).[13] Our study population being predominantly from North India had a frequency of 25% *EGFR* positivity which corroborates with their findings. Similar observation was found in previous studies from our institute.[14,15]

KRAS is the most frequently mutated gene across all cancer types with varying frequency in different regions of the world. The percentage of *KRAS* mutations in NSCLC observed in Western countries varies from 11% to 38% where Austria shows maximum frequency.[16-18] In Asian countries, the range is much lower and constitutes only 3% to 19% in all NSCLCs.[9,10,19-23] Two previous studies from India, both from the same center have revealed lower frequencies of *KRAS* positivity of 6.4% and 1.5% respectively[7,24]; as compared to 19.5% in the present study. The primary reason for this variation can be attributed to the method of detection of the mutation and higher prevalence of smokers in this cohort. Real-time PCR -based kits used in the present study have better sensitivity and specificity as compared to Sanger sequencing used in other studies.

Biologically, *BRAF* alterations are associated with increased kinase activity thereby rendering constitutive activation of the MAP kinase pathway. The percentage of *BRAF* mutation in NSCLC observed across the globe varies from 0.4% to 4.9% in the Western countries[16,18] to 0.3% to 1.9% in Asia.[7,22-24] Our findings are in concordance with global data. The targetable mutations which have been identified are *BRAF* L597V and V600E point mutations. Two of our cases had L597V point mutation while the V600E point mutation was absent in our cohort. Other *BRAF* mutations like G446D, G4469A/L, and Y472C have been identified but lack therapeutic trials. The third *BRAF* positive case in our study exhibited G442D point mutation. This mutation has earlier been reported in colon cancers[25] and proven to be the causative factor.[26,27] Additionally, we also observed *BRAF* polymorphism in 14.4% cases. However, the clinical implications of *BRAF* G442D point mutation and polymorphism require further exploration in NSCLC.

We had twice the population of males and approximately 70% of patients were smokers. *KRAS* mutations are known to be common in males and smokers.[9,22,28,29] On the other hand, we differed in the gender distribution and smoking status of *EGFR* and *BRAF* positive cases.[9,30-32]

The overall predominance of male smokers in our cohort may be a confounding factor for the above results.

EGFR mutations are strongly associated with acinar, lepidic and papillary subtypes and are rarely found in mucinous.[32-36] In contrast, the association of *KRAS* and *BRAF* mutations with histologic subtypes is controversial.[33] Multiple reports have shown *KRAS* mutations to be prominent in invasive mucinous ADCA, whereas others have reported them to be associated with the solid subtypes.[33,36-40] We found significant correlation of *EGFR* mutation with acinar, lepidic and papillary patterns; however, *KRAS* mutations were not associated with mucinous histology ($P = 0.036$).

The treatment modality offered to our patients solely depended on the clinicians' discretion keeping in mind the ECOG performance status, stage, and the mutation profile available at the time of treatment decision making. We estimated the PFS in all cases with available follow-up post-treatment. The median PFS of *EGFR* mutated and wild type patients were almost similar. This may be due to the fact that some patients had not received TKI despite being *EGFR* mutated. Effective therapy targeting *KRAS* mutation in lung cancer has not been developed yet. Studies on PFS of *KRAS*-mutated lung ADCA, irrespective of mode of treatment, also contradict each other.[20,41-44] PFS of *KRAS* wild type and mutated patients in our study differed marginally.

One area requiring further research is the co-existence of multiple exonic mutations in a single gene in both *EGFR* as well as *KRAS* genes. We had nearly 4% of patients harboring multiple mutations in single gene. All of them were in advanced clinical stage and had no significant association with smoking. Various clinical trials and studies have reported incidence of *EGFR* multiple mutations varying from 0.47% to 2.1%[9,45] with poorer response rate.[45] We had only 1 case with multiple mutations in the *EGFR* gene whose survival status could not be determined as he was lost to follow-up. A unique finding was the detection of *KRAS* multiple mutations, which has not been reported in lung cancers. Those conducted in colorectal cancers have shown an incidence of 2.1% and are seen to be associated with advanced clinical stage and metastases.[46] We had 4 *KRAS* mutated cases with multiple mutations. All 4 had received conventional chemotherapy and had shown variable treatment response. Due to the small number of cases with *KRAS* multiple mutations, it is difficult to draw any conclusion about the treatment response and survival characteristics of such patients, needing further extensive studies to address the issue.

Besides the presence of multiple mutations in the same gene, coexistence of multiple gene mutations is also known with incidence rates up to 5%.[47-50] The demographic profile of such patients has not been described earlier. Our patients with such mutational profile were predominantly males in the older age group and all were in the advanced clinical stage. The coexistence of mutations in a patient may be a consequence of intratumoral heterogeneity due to coexisting divergent clones within the same tumor.[49,50] Tumors with *EGFR* and *KRAS* co-positivity generally show histologic features typical of *EGFR* positive cases (predominant acinar pattern).[49] Similarly, 3 of our 4 cases with dual positivity had acinar histology. Further, it has been hypothesized that presence of coexisting mutations is associated with an aggressive disease profile with suboptimal response to therapy. We had follow-up available in 2 cases with coexisting mutations, both of which had initial short lasting clinical response to TKI and subsequently progressed (approximate PFS was 5 months). One of them was later found to have developed T790M mutation after treatment with *EGFR* TKI. Presence of co-existing mutations challenges the concept of mutual exclusivity and highlights intratumoral heterogeneity which may be helpful in predicting clinical response to targeted therapy.

On comparing the PFS status of patients with single mutations or wild type status with those having multiple or coexisting mutations, the difference was of only 1 month and was not statistically significant ($P = 0.09$). However, the P value of 0.09 may indicate a borderline correlation and extensive follow-up of such patients is warranted in further studies.

Also we are unable to comment on the benefits of different therapeutic regimens (TKI vs chemotherapy) in the different mutant groups due to minimal number of cases with satisfactory follow-up in each subgroup.

To conclude, this study examined the genetic alterations of key genes involved in MAP kinase pathway in lung ADCA among Indians. In addition, the study highlights the importance of co-existence of driver mutations in lung cancer which may have clinical implications on disease progression and therapeutic responses. We also found that, not only *EGFR* but *KRAS* also exhibited multiple mutations in lung ADCA, a finding which needs further exploration. Lung ADCA commonly exhibits non-V600E *BRAF* mutations. Finally, we reiterate the importance of testing of all major genetic driver mutations in lung ADCA irrespective of histology, smoking status, and other demographic characteristics.

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