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Original Article

Pathology Section

Phenotypic and Genotypic Appraisal of Pulmonary Mucinous Adenocarcinoma: A Retrospective Study

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ABSTRACT

Introduction: Lung cancer is the second most common cancer diagnosed in the world. According to GLOBOCAN 2020, it is approximately 11.4% of all malignancies and accounts for one in five cancer deaths worldwide. Several driver gene mutations are known in lung adenocarcinoma including Epidermal Growth Factor Receptor (EGFR), Kirsten rat sarcoma viral oncogene homolog(KRAS), v-raf murine sarcoma viral oncogene homolog B1(BRAF), Human epidermal growth factor receptor2 (HER 2), anaplastic lymphoma kinase (ALK), Ros Protooncogene 1, Receptor Tyrosine Kinase (ROS1) and rearranged during transfection (RET). Among these, KRAS mutation has been classically defined as bearing a negative prognostic factor. They are described to have unfavourable survival rates as compared with KRAS wild- type tumours. However, its real significance remains controversial due to heterogeneity amongst studies.

Aim: The aim of this study was to assess the histomorphology, immunophenotype, EGFR and KRAS mutation profile in invasive mucinous and mixed mucinous/non mucinous type lung carcinoma.

Materials and Methods: This retrospective study was done at a tertiary cancer centre in the state of Kerala, South India to identify the frequency of EGFR and KRAS mutations in invasive mucinous or mixed mucinous/non mucinous type lung carcinoma diagnosed during the period June 2017 to March 2019. Immunohistochemical assays for CK7, CK20, TTF-1, Napsin-A, CDX-2 and p63 were done on all cases. EGFR mutation analysis for exons 18, 19, 20, 21 were done using ARMS PCR and KRAS mutation analysis for codons 12, 13, 61, 146 by Sanger sequencer. Descriptive statistics was used wherever applicable.

Results: Out of a total of 290 patients of pulmonary adenocarcinoma during the two year study period, twelve cases (4.1%) were diagnosed as invasive mucinous or mixed mucinous/non mucinous types based on histomorphology and immunophenotype, on both lung tissue biopsies as well as material from metastatic sites. Of the twelve, nine (75%) were pure mucinous and three (25%) were mixed mucinous and non mucinous. All the twelve cases were subjected to molecular testing for both EGFR and KRAS. Three cases (25%) showed KRAS mutations in codon 12. EGFR mutation (TKI resistant T790M mutation) was found only in a single case which had a histology of mixed mucinous and non mucinous carcinoma and a coexistent mutation in KRAS (c12 G>C) gene.

Conclusions: Our study corroborates the described fact that pulmonary mucinous carcinoma differ from the conventional adenocarcinoma in terms of its histology, immunoprofile and molecular profile. KRAS mutation appears to be the main pathogenetic factor in this variant. In the current day practice, there is no demonstrable effective therapy for KRAS mutant lung adenocarcinoma and more trials are needed to identify newer mutations that can modify treatment outcomes.

Keywords: Histomorphology, Immunophenotype, Kirsten rat sarcoma viral oncogene homolog mutations, Lung Adenocarcinoma

INTRODUCTION

Lung cancer is recognised as a leading cause of cancer related mortality in men. Among cancers affecting women, it ranks third in incidence and second in mortality [1]. It is noteworthy that incidence and mortality rates are roughly two times higher in men than in women, although the male-to-female ratio varies widely across regions. Two-thirds of lung cancer worldwide is attributed to smoking tobacco and hence interventions to reduce tobacco usage are very much the need of the hour [1]. Outdoor air pollution, ionizing radiation and other inhalable biohazard agents are also implicated in the pathogenesis of lung cancer. Over the years, a shift in the major histologic subtype of lung cancer was observed with a relative increase in frequency of adenocarcinoma compared to squamous and small cell types. Invasive adenocarcinoma is a malignant epithelial tumour with glandular differentiation, mucin production or pneumocyte marker expression [2]. Invasive Mucinous Adenocarcinoma (IMA) are those with tumour cells having goblet cell/columnar morphology and intracytoplasmic mucin (formerly classified as mucinous bronchioloalveolar carcinoma) and with a distinct immunoprofile of CK7 & CK20 positive and TTF-1 & NapsinA negative [2]. Several driver gene mutations are recognised in lung adenocarcinoma, including EGFR, KRAS, BRAF, HER2, ALK, ROS1 and RET. Each of these genetic alterations is associated with specific clinical characteristics, pathological features, prognostic and predictive features. Previous studies have reported these mutations as mutually exclusive. EGFR is a member of the transmembrane receptor family whose mutations influences the growth factor signaling pathway, leading to carcinogenesis and metastases. Mutations in EGFR gene is most common in Non Small Cell Cancer (NSCC) with histology of adenocarcinoma, in those who have never smoked and female patients of Asian ethnicity [3-6]. In tumours with sensitizing mutations in EGFR, first line of treatment with Tyrosine Kinase Inhibitors (TKI) is associated with high overall response rate, longer Progression Free Survival (PFS) and better quality of life compared with patients with chemotherapy alone [4]. KRAS is a member of the canonical RAS family of genes. KRAS mutation has been found to occur at higher frequency in smokers compared to those non smokers and is described in mucinous adenocarcinoma [3]. Although the KRAS-MAP Kinase pathway is a downstream of EGFR signaling, KRAS mutation driven lung adenocarcinoma, do

not respond to Tyrosine kinase inhibitors and effective targeting therapies are yet to be developed [7]. KRAS has also been found to be negative predictor for TKI therapy. Although KRAS mutations have been classically defined as a negative prognostic factor compared to KRAS wild-type tumours, its real significance remains controversial due to heterogeneity amongst studies [5].

To date, no efforts at targeting KRAS have been proven successful and most patients with KRAS mutated tumours are treated with chemotherapy regimens based on platinum. This study aimed to assess KRAS mutation profile in pulmonary adenocarcinoma with histomorphology and immunoprofile of invasive mucinous or mixed mucinous/non mucinous type.

MATERIALS AND METHODS

A retrospective analysis of cases reported as mucinous and mixed mucinous/non mucinous adenocarcinoma was conducted during a two year period from June 2017 to March 2019 at a tertiary cancer care centre. The study protocol was approved by the Institutional Review Board (1616/IRB-SRC/13/MCC/11-05-2019/6 dated 23rd May 2019) and conducted during the period June to December 2019.

Inclusion criteria: All cases of pulmonary mucinous adenocarcinoma (>10% mucinous component) and mixed mucinous with non mucinous carcinoma were included in the study.

Exclusion criteria: Cases of pure non mucinous adenocarcinoma were excluded from the study.

Demographic data like age, sex, tumour laterality, tumour site, type of biopsy were retrieved from medical records and the haematoxylin and eosin stained slides were reviewed. Histological subtyping was performed as per International Association of study of Lung Cancer/ American thoracic society/European respiratory society (IASLC/ ATS/ERS) and was divided into two groups: pure mucinous (>90% mucinous pattern) and mixed mucinous/non mucinous (> 10% of each component) [2].

Formalin Fixed Paraffin Embedded (FFPE) tumour specimens of the twelve cases were subjected to immunoperoxidase staining for Cytokeratin 7, Cytokeratin 20, TTF-1, Napsin-A, CDX-2 and p63 (DAKO monoclonal antibodies) using automated stainer (Bond Max, Leica Biosystems). Diaminobenzidine was used as the chromogen and haematoxylin as the nuclear counterstain. Positive tissue controls for each immune marker were stained in parallel with the study cases. Presence of membrane staining for cytokeratins, nuclear staining for TTF-1, p63 and CDX2 and cytoplasmic staining for Napsin A were interpreted as positive.

All cases were subjected to EGFR and KRAS mutation analysis. EGFR mutation analysis was performed for exons 18, 19, 20, 21 using amplification refractory mutation system polymerase chain reaction (ARMS PCR), using taqman based PCR kit. KRAS mutation analysis for codons 12, 13, 61, 146 was performed by Sanger sequencer. Exon 2,3 4 were amplified using following primer sets (a) exon 2 Forward Primer-GAGTGAACATCATGGACCCTCACA, Reverse Primer -TTAAGCGTCGATGGAGGAGTTTG; (b) exon 3 Forward CCAACTGTGTTTCTCCCTTCC Reverse Primer CTATAATTACTCCTTAATGTCAGC (c) exon 4 Forward TGGACAGGTTTTGAAAGATATTTGT Reverse Primer ATTAAGAAGCAATGCCCTCTCAAG. Amplified products were purified using the QIAquick Gel Extraction Kit (QIAGEN) as per manufacture protocol. Cycle sequencing was performed using BigDye™ Terminator v3.1 Cycle Sequencing Kit (applied biosystems) with either forward for reverse primer using standard cycle sequencing condition. Cycle sequenced product was purified using ethanol/EDTA precipitation protocol as mentioned in the user manual of BigDye™ Terminator v3.1 Cycle Sequencing Kit. Further resuspend purified and dried sequencing reactions in 10-µL of Hi-Di™ Formamide. A 3500 genetic analyser was used to run the resuspend product, data analysed using sequence analysis software. Genetic variant was detected using the BLAST analysis.

STATISTICAL ANALYSIS

Data entry and statistical analysis was done using MS excel. Categorical variables were summarised using counts and percentages. Continuous variables were summarised using mean and standard deviation.

RESULTS

Out of a total of 290 patients of pulmonary adenocarcinoma during the two year study period, there were twelve cases (4.1%) diagnosed as invasive mucinous or mixed mucinous/non mucinous types based on histomorphology and immunophenotype, on both lung tissue as well as material from metastatic sites. Mean age of the patients was 56 years. More than half of the patients were males (7, 59%). The tissue material varied from lung resection (1, 8.3%), core biopsies (4, 33%), lymph node excisions (3, 25%) or pleural biopsies (4, 33%). Of the twelve, nine (75%) were broadly classified as pure mucinous and three (25%) were mixed mucinous and non mucinous. Final histologic subtypes were seven cases (59%) of pure invasive mutinous adenocarcinoma, four cases (33%) of mixed mucinous and non mucinous and one case of enteric (1, 8.3%) [Table/Fig-1]. The immune and molecular profiles of the study cases are mentioned in [Table/Fig-2]. The immunoprofile was based on staining for the markers cytokeratins 7, 20, TTF1, Napsin A, CDX2 and p63. Cytokeratin 7 was positive in all cases (8/8,100%), cytokeratin 20 in 43% (3/7), Napsin A in 40% (2/5) and TTF in 58% (7/12) [Table/Fig-3]. p 63 and CDX2 were negative in the single case each where it was done.

		Frequency	Percentage
Total cases		12	100
Type of sample	Resection	1	8.3
	Core biopsies	4	33.3
	Lymph node excision	3	25
	Pleural tissue	4	33.3
Histological subtypes	Pure mutinous adenocarcinoma	7	58.3
	Mixed mucinous and non mucinous adenocarcinoma	4	33.3
	Enteric	1	8.3

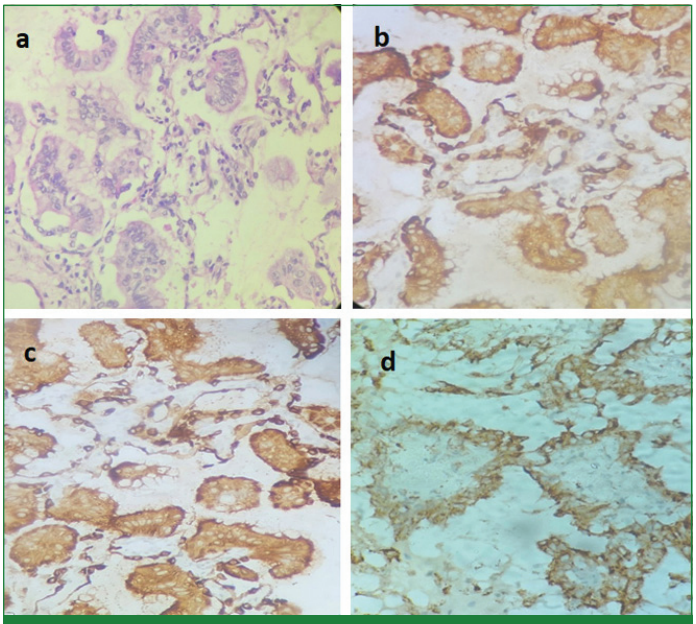
[Table/Fig-1]: Type of sample and histologic subtypes of study cases.

		Positive		Negative		Not done		Reason not done
Test	**Markers**	**n**	**%**	**n**	**%**	**n**	**%**	
Immunostain	TTF-1	7	58.3	5	41.6	0	0	
	Napsin A	2	16.6	3	25	7	58.3	Suboptimal tumour volume
	Cytokeratin 20	3	25	4	33.3	5	41.6	Suboptimal tumour volume
	Cytokeratin 7	8	66.6	1	8.3	3	33.3	Suboptimal tumour volume
Molecular	KRAS	3	25	5	41.6	4	33.3	Suboptimal tumour volume
	EGFR	1	8.3	11	91.6	0	0	

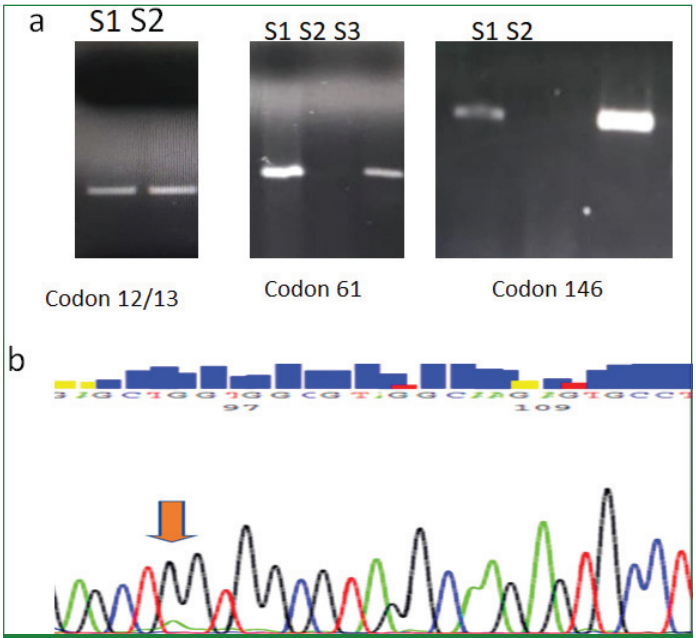
[Table/Fig-2]: Immuno and molecular work-up of the study cases.

Out of the twelve cases, three (25 %) revealed KRAS codon 12 mutations, five (42%) did not reveal any mutation in codons 12, 13 and 146 (PCR amplification for region spanning codon 61 failed for one case) and three (25%) were inadequate for analysis. KRAS mutation positive cases revealed c.35G>A (p.Gly12Asp) in two cases and c.35G>C (p.Gly12Ala) in one case [Table/Fig-4]. On EGFR mutation analysis by ARMS PCR, only a single case showed

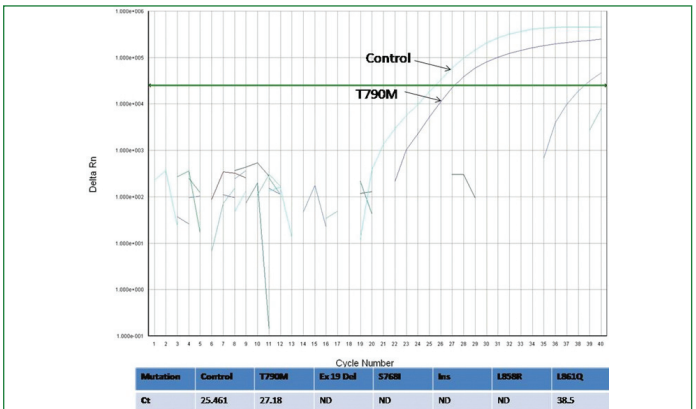
a mutation, namely a resistant mutation T790M. This one case had also revealed KRAS mutation on Sanger sequencer [Table/Fig-5].



[Table/Fig-3]: (a) Microphotograph showing neoplastic glands lined by tall columnar cells with mucin filled cytoplasm H&E(40x); (b) Immunohistochemical stain showing positivity for cytokeratin 7 in neoplastic cells (40x); (c) Immunohistochemical stain showing positivity for cytokeratin 20 in neoplastic cells (40x); (d) Immunohistochemical stain showing positivity for Napsin A in neoplastic cells (40x).



[Table/Fig-4]: (a) PCR amplification of the KRAS region for sequencing the codons12,13,61 and146; (b)Electrophorogram shows KRAS mutation c.34C>T;p.12Gly>Ser9 (as depicted by the arrow).



[Table/Fig-5]:](a) Real Time PCR amplification image of EGFR mutation analysis, Sample showing the T790M mutation. Mutant Ct (threshold cycle)- control Ct less than 7.6 was taken as positive.

DISCUSSION

Classification of the histological subtypes as proposed by IASLC/ATS/ERS clearly elucidates the morphology, immunoprofile, genetics and prognostic significance of the different subtypes [2]. Mutations in genes like EGFR, KRAS, ALK, ROS are considered the prototype driver mutations in lung cancers. The use of TKIs in EGFR mutated adenocarcinoma is well established. Activating somatic mutations in the EGFR gene conferring sensitivity to EGFR TKIs were first reported in 2004 and mutations like exon 19 deletion and L858R mutation in exon 21 are predictive of response to the EGFR TKIs, gefitinib and erlotinib. Several different KRAS mutations can lead to non small cell lung cancer, and the most commonly identified are the transition mutations (G→A) and transversion mutations (G→T or G→C). It is present in approximately 10% of lung adenocarcinoma. KRAS mutations are also sometimes found in other types of cancer, including pancreatic, colon, endometrial, bile duct, and small intestine cancers. It is well known that treatment response in cancer depend on the genetic features of the tumour. Treatment is difficult for the cancer cases harbouring KRAS activating mutation compare to KRA wild type. Such cases of KRAS mutation can be referred for new clinical trials that target this abnormality. Patients whose tumours do not have mutations in either EGFR or KRAS may have another abnormality involving the ALK gene. Patients with ALK mutation can respond to targeted treatment with crizotinib.

IMA differs from the usual subtypes of lung adenocarcinoma in its biology, morphology, immunoprofile and genotype and is described as a new entity in the IASLC/ATS/ERS. The former name was mucinous bronchioloalveolar carcinoma. Tumour cells are columnar or appear like goblet cell filled mucin filled cytoplasm and small basal nuclei. In contrast to the other subtypes, the cells are positive for cytokeratin 20 and negative for TTF and Napsin [3]. This study reports on the phenotype and genotype of such cases as fits the criteria defined by WHO.

In this series, predominant histology was IMA (7,58%) followed by MMANM (4,33%). More than half were positive for CK 20 (57%). Napsin A and TTF-1 were not expressed in 60% and 41%, respectively. The cases with positive staining for NapsinA and TTF-1 were MMANM which is explainable give its non mucinous component.

The selection of patients with lung cancer for molecular testing is currently based on recommendations from the CAP/ IASLC/AMP and includes testing for EGFR, KRAS, ALK and ROS [5,8,9]. The guidelines of the professional societies like IASLC, CAP, AMP and National Comprehensive Cancer Network (NCCN) recommends that molecular testing be made a routine diagnostic offer to patients with advanced non small cell lung cancer [10]. All the twelve cases in this study had the histomorphology and immunoprofile of Invasive Mucinous Carcinoma (IMA) or Mixed Mucinous-Nonmucinous Adenocarcinoma (MMNMA). All cases of IMA or MMNMA were subjected to molecular testing for both EGFR and KRAS. Three cases of IMA showed KRAS mutations in codon 12 and wild type EGFR; one case which had the histology of MMNMA showed mutations in both EGFR (TKI resistant T790M mutation) and KRAS(c12 G>C) genes.

Literature review has revealed studies with similar findings. Kadota K et al., found 61% of KRAS mutations which were more commonly detected in pure mucinous adenocarcinoma (85%) as compared to mixed mucinous and non mucious carcinomas (31%) [6].

Lu F et al., reported a 5% incidence of IMA and 35.3% frequency of KRAS mutation in a cohort of 269 patients (p=.006) [11]. Some studies like Dong YJ et al., reported a higher frequency of IMA (24.5%) and EGFR was detected in 23.4% of these cases. Here KRAS mutations were characterized by adenocarcinoma with

mucin production (19.1%) (p=.008) [6]. In a study by Yoshizawa A et al., conducted in 440 Japanese patients, 2.2% of the cases were IMA. EGFR was not detected in mucinous adenocarcinoma; whereas KRAS was detected in solid type adenocarcinoma (25.0%) and in all IMA [12].

In a NGS based analysis of IMA, mixed carcinomas and adenocarcinoma with mucinous features, Hwang DH et al., detected that 71% of KRAS mutation in codons12 or 61 [13]. It thus seems that KRAS mutations are significantly associated with invasive mucinous and solid types [14].

Limitation(s)

The study is limited by the small sample size of the specific histologic subtype of IMA to conclude with significant statistical correlation. Another limitation was the difficulty in DNA extraction from FFPE leading to poor quality or inadequacy of genetic material for mutation testing. Often the tumour cells in small core biopsies were insufficient for phenotypic and genotypic analysis.

CONCLUSION(S)

Absence of detectable EGFR mutation and presence of KRAS mutation in three cases of pure IMA confirms the latter as the possible driver mutation. The exact biology of this specific variant is yet to be fully understood and more trials are needed to understand the pattern of the driver mutation. In the current day practice, there is no demonstrable effective therapy for KRAS mutant lung adenocarcinoma. With increasing availability of targeted therapies against specific molecular defects, these findings offer new opportunities for personalised cancer care.

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