Original Article

Profile of IHC Marker in
Lung Tumours

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ABSTRACT

Background: Lung biopsy is crucial in histopathological subtyping and classification. However, making an accurate diagnosis in small biopsies can be challenging. This study was aimed to distinguish and classify lung tumors employing immunohistochemical markers TTF-1(thyroid transcription factor 1), Cytokeratin 5/6, Cytokeratin 20, AE1/3. Subsequently prognostic value of different histological types of lung tumours was established employing Ki-67.

Material & Methods: A total of 52 clinically diagnosed cases of lung carcinoma over a period of two years were included in our study. Of these 2 cases were excluded from the study as they showed granulomas on histopathology. All the cases after being diagnosed on H&E were subjected to Immuohistochemistry (IHC).

Statistical Analysis: The statistical analysis was done using SPSS for Windows 15.0 program. Specificity, sensitivity, positive predictive value, negative predictive value of all these IHC markers was statistically evaluated.

Results: Of 29 cases diagnosed as Squamous cell carcinoma (SCC) on H&E, 26 were CK5/6 positive on IHC. Of 4 cases diagnosed as Primary Adenocarcinoma (ADC) on H&E, 3 were TTF-1 positive. Of 4 cases diagnosed as poorly differentiated carcinoma on H&E, 1 case was diagnosed as SCC and 2 cases as Metastatic ADC on employing IHC markers. Ki-67 was high in 82.8% cases of SCC and in 50% cases of ADC. The sensitivity and specificity of CK5/6 and TTF-1 was 100%, 57.1% and 100%, 96.7% respectively.

Conclusion: Employing IHC markers either singly or in a panel is a useful adjunct to morphological features and clinical parameter in the diagnosis and management of lung tumour.

Keywords: Adenocarcinoma, CK5/6, Immunohistochemistry, Squamous cell carcinoma, TTF-1

Introduction

Lung cancer is the leading cause of cancer related mortality worldwide. [1,2] In India, lung cancer constitutes 6.9% of all new cancer cases and 9.3% of all cancer related deaths in both sexes.[3] Primary lung carcinomas have been divided into small cell lung carcinomas (SCLC) and non-small cell lung carcinomas (NSCLC). The main histologic types of NSCLC are adenocarcinomas (50-70%), squmous cell carcinoma(20-30%) and large celll carcinoma(<10%).[4,5]

Majority of non-small cell lung carcinomas (NSCLC) can be diagnosed and further subtyped employing hematoxylin and eosin (H&E) stains. However, morphological recognition of poorly differentiated tumours may be difficult, especially in smalll biopsies.[6] To accurately diagnose these tumours there is an increasing demand of ancillary techniques such as IHC. Furthermore, IHC plays an important role in distinguishing metastatic lesion from primary lung carcinomas.[7] Because of underlying difficulties to rely on single IHC marker, a panel of markers is suggested to improve sensitivity and specficity.

In squamous cell carcinoma p63 and Cytokeratin 5/6 are commonly used markers while TTF-1 and Napsin A have been proven to stain majority of lung adenocarcinomas. [8, 9] Thus, this study was undertaken with an aim to identify a panel of IHC markers that can accurately distinguish Squamous cell carcinoma from Adenocarcinoma, tumours of epithelial origin from lymphoid origin and primary lung adenocarcinoma from metastatic adenocarcinoma.

Materials and Methods

This study was carried out in the Department of Pathologyin a tertiary care hospital in Uttarakhand. Approval of the study was taken from the institutional ethical committee before commencing the study.

It was a prospective study. All cases that were clinically diagnosed as lung tumour during a period of 20 months (October2015- July 2017) were included in the study. Total 52 clinically diagnosed cases of lung tumours were included in this study. Relevant history, clinical findings and radiological reports were recorded on the designated pre-tested proforma.

Tissue samples were received as bronchial or pleural biopsies in 10% formalin. Grossing was done as per the protocol of histopathology laboratory. All the formalin fixed tissues were processed in automatic tissue (Shandon Citadel 2000). The blocks were made from processed tissue using Embedding station (Leica EG1150h+C).

Sections of 3-5μ were made using microtome. They were stained by haematoxylin and eosin, and mounted in DPX. The sections were studied using light microscope and detailed histopatholgy report was done by consultant histopathologist.

Immunostaining: For IHC, additional 5 sections of 2-4 microns from the above paraffin embedded blocks were made and taken on poly-L lysine coated slides. They were subjected to immunohistochemistry employing TTF-1 for Adencarcinoma, CK5/6 for SCC, CK20 to differentiate primary lung adenocarcinoma from metastatic Adenocarcinoma and immuno-staining for pan-cytokeratin, AE1/AE3 to demonstrates that the tumor is a carcinoma rather than a lymphoid tumor using primary antibodies DO7 (1/50 Dako) and Mib-1 (1/50 Novocastra). Sections were incubated with the secondary biotinylated antibody and avidin – biotin peroxidase complexes for 30 minutes. Reaction products were revealed with diminobenzidine (DAB) as the chromogen and sections were counterstained with Harri's hematoxylin to enhance nuclear detection.

Sections of normal thyroid tissue that was detected for TTF-1 and achronic tonsillitis that was detected for ki-67 was used as positive controls. Sections of normal skin tissue that was detected for CK5/6, CK20 and AE1/3 was used as positive control. For negative controls, primary antibody was substituted with phosphate buffer saline (PBS) in duplicate sections.

Interpretation: The staining intensity and area of sections were evaluated under low magnification(X 20). Histologic (H) scores were conducted following the recognised method for CK5/6, AE1/3, TTF-1 and CK20. A 4 level scoring for intensity was used (0 negative; 1+, weak; 2+moderate; 3+strong). The expression intensity (0-300) was determined by multiplying the scores by the percentage of positive area (0-100%). Sections with H score of >150 were considered to show a high expression, and those with H scores of ≤150 were considered to show a low expression. [10]

For calculating the ki- index, the entire section was screened to find the region with maximum number of tumour cells showing positivity. Percentage of tumour cells that were positive was calculated by counting atleast 100 cells. The immunohistochemical results were interpretated according to the intensity of immunoreactive product seen in the nuclei of tumour cells.

Statistical analysis: The statistical analysis was done using SPSS for Windows 15.0 program. The sensitivity, specificity, PPV, NPV of markers CK5/6, TTF-1, CK20, AE1/3 for all the cases was calculated using Galan & Gambino Method. Fisher's Extract Test was performed to compare categorical variables with status of CK5/6 and TTF-1 expression. A p value of <0.05 was accepted as statistically significant.[11]

Results

Of all the 52 cases that were clinically diagnosed as lung carcinomas, the M:F ratio was 4.6:1 with maximum number of cases seen in the age group 61-80 years(60%). The commonest presenting symptom in all these cases was fever with cough (40%, 20 cases). Of all the 52 cases, a histopathologic diagnosis could be made in 42 cases (80.8%). Of the remaining 10 cases, 4 cases were diagnosed as suspicious of malignancy with no confirmed histopathological category and 6 cases were grouped in the category of others. Of these 6 cases, 4 cases showed epithelial dysplasia and were included in the study while the other 2 cases showed necrotising granulomas on histopathology and were excluded from the study.

Histologic correlation of 50 cases of lung tumours included in the study with distribution pattern of all the IHC markers was done. (Table1)

Out of 29 cases of Squamous cell carcinoma diagnosed on H&E (fig.1A), 26 cases (89.7%) were positive for CK5/6 thus confirmed as squamous cell carcinoma (Fig.1B,C). All the 29 cases were negative for TTF-1 & CK20. It was observed that out of 29 cases of SCC, 24 cases (82.8%) showed high ki-67 index(1D). Out of 5 cases adencarcinoma diagnosed on H&E, 4 cases (80%) were positive for TTF-1 and one case (20%) was positive for CK20. All the 5 cases were negative for CK5/6 but were positive for AE1/3. Hence, out of 5 cases diagnosed as Adenocarcinoma on H&E, 4 cases were confirmed as primary adenocarcinoma while 1 case was confirmed as metastatic adenocarcinoma on IHC. A high Ki67 index was observed in 3 cases (60%) out of 5 cases of Adenocarcinoma.

Out of 4 cases diagnosed as poorly differentiated carcinoma on H&E (Fig.2A), all the 4 cases were confirmed of epithelial origin as they were positive for AE1/3. One case (25%) exhibited positivity for CK5/6 hence confirmed as Squamous cell carcinoma and 2 cases exhibited positivity for CK20 thus were confirmed as metastatic adenocarcinoma (Fig 2B,C). None of the cases were positive for TTF-1(Fig. 2D). A high expression of ki67 (50%) was seen in 2 cases out of 4 cases.

Out of 4 cases diagnosed as Small cell carcinoma on H&E, 2 cases (50%) were positive for AE1/3 thus confirming the epithelial origin. Of these, 1 case showed positive expression for TTF-1 and was confirmed as Small cell carcinoma(Fig. 3A,B),. None of the case exhibited a positive expression for either CK5/6 or CK20. Ki67 expression was high only in 2 cases.

On histopathology, 4 cases were categorised as Suggestive/ Suspicious of malignancy. Of these, 3 cases were positive for AE1/3 thus confirming the epithelial origin of tumour. Out of these 3 cases, 2 cases (50%) exhibited a positive expression for CK5/6 thus confirming it to be Squamous Cell carcinoma (Fig.3C,D) and 1 showed a positive expression for TTF-1 thus confirming a diagnosis of Adenocarcinoma. One case showed no positive expression for either markers. A high expression of Ki67 was seen only in 1 case (>25%).

Four cases showing epithelial dysplasia with atypical cells were grouped in the category of Others. Of these 4 cases,

only 1 case (25%) exhibited a positive expression for AE1/3 and CK5/6 and was labelled as Squamous Cell Carcinoma. The remaining 3 cases in this category were negative for all the markers and a repeat biopsy from representative area was asked on account of high degree of suspicion.

For Squamous cell carcinoma, Sensitivity, Specificity, PPV, NPV and accuracy for CK5/6 was 86.6%, 85%, 89.6%, 81.0% and 69% respectively. Immunostaining results for sensitivity, specificity, PPV, NPV and Accuracy for TTF1 in Adenocarcinoma were 50%, 97.7%, 75%, 93.5% and 92.0% respectively. The overall diagnostic Accuracy of CK5/6, TTF-1 AE1/3&CK20 was 60%,92%,82% and 92% respectively (Table 2). H score was calculated for all the markers (Table 3).

Discussion

The present study was conducted on bronchial and or pleural with regards to ability to diagnose and differentiate non-squamous and squamous cell carcinomas. Differentiating

Table 1: Showing distribution pattern of all the IHC markers in all the 50 cases of lung tumors.

Typing	CK5/6	TTF-1	AE1/3	CK20	Ki67(>20%)
SCC	26(89.7%)	0	29(100%)	0	24(82.8%)
ADC	0	4(80%)	5(100%)	1(20%)	3(60%)
Poorly differentiated carcinoma	1(25%)	0	4(100%)	2(50%)	2(50%)
Small cell carcinoma	0	0	2(50%)	0	2(50%)
Suspicious of malignancy	2(50%)	1(25%)	3(75%)	0	1(25%)
Others	1(25%)	0	1(25%)	0	0

Table 2: Shows sensitivity, specificity, positive predictive value, negative predictive Value and Diagnostic Accuracy of all the 4 markers in all the 50 cases.

Varibales	Sensitivity	Specificity	PPV	PPV	Accuracy
CK 5/6	100.0%	57.1%	89.7%	100.0%	90.9%
TTF-1	100.0%	96.7%	75.0%	100.0%	97.0%
AE1/3	100.0%	0.0%	100.0%	0.0%	100.0%
CK20	0.0%	100.0%	0.0%	97.0%	97.0%

Table 3: H score

H score					
Diagnosis		CK 5/6	TTF-1	AE1/3	CK20
Squamous cell carcinoma	N	29	28	28	21
	Mean	111.379	0.000	242.536	0.000
	SD	69.7815	0.0000	38.9876	0.0000
	Median	135.000	0.000	247.500	0.000
Adenocarcinoma	N	4	4	4	3
	Mean	0.000	100.000	217.500	70.000
	SD	0.0000	70.7107	28.7228	121.2436
	Median	0.000	125.000	225.000	0.000
Metastatic Adenocarcinoma	N	1	1	1	1
	Mean	0.000	210.000	210.000	0.000
	SD				
	Median	0.000	210.000	210.000	0.000

| H score					
Diagnosis		**CK 5/6**	**TTF-1**	**AE1/3**	**CK20**
Others	N	4	4	4	3
	Mean	20.000	0.000	123.750	0.000
	SD	40.0000	0.0000	62.8987	0.0000
	Median	0.000	0.000	150.000	0.000
Poorly differentiated carcinoma	N	4	4	4	3
	Mean	45.000	0.000	208.750	140.000
	SD	90.0000	0.0000	72.8440	121.2436
	Median	0.000	0.000	240.000	210.000
Small cell carcinoma	N	4	4	4	4
	Mean	0.000	45.000	97.500	0.000
	SD	0.0000	90.0000	113.2475	0.0000
	Median	0.000	0.000	90.000	0.000
Suggestive/Suspicious of Malignancy	N	4	4	4	1
	Mean	72.500	45.000	221.250	0.000
	SD	83.8153	90.0000	33.2603	
	Median	70.000	0.000	225.000	0.000
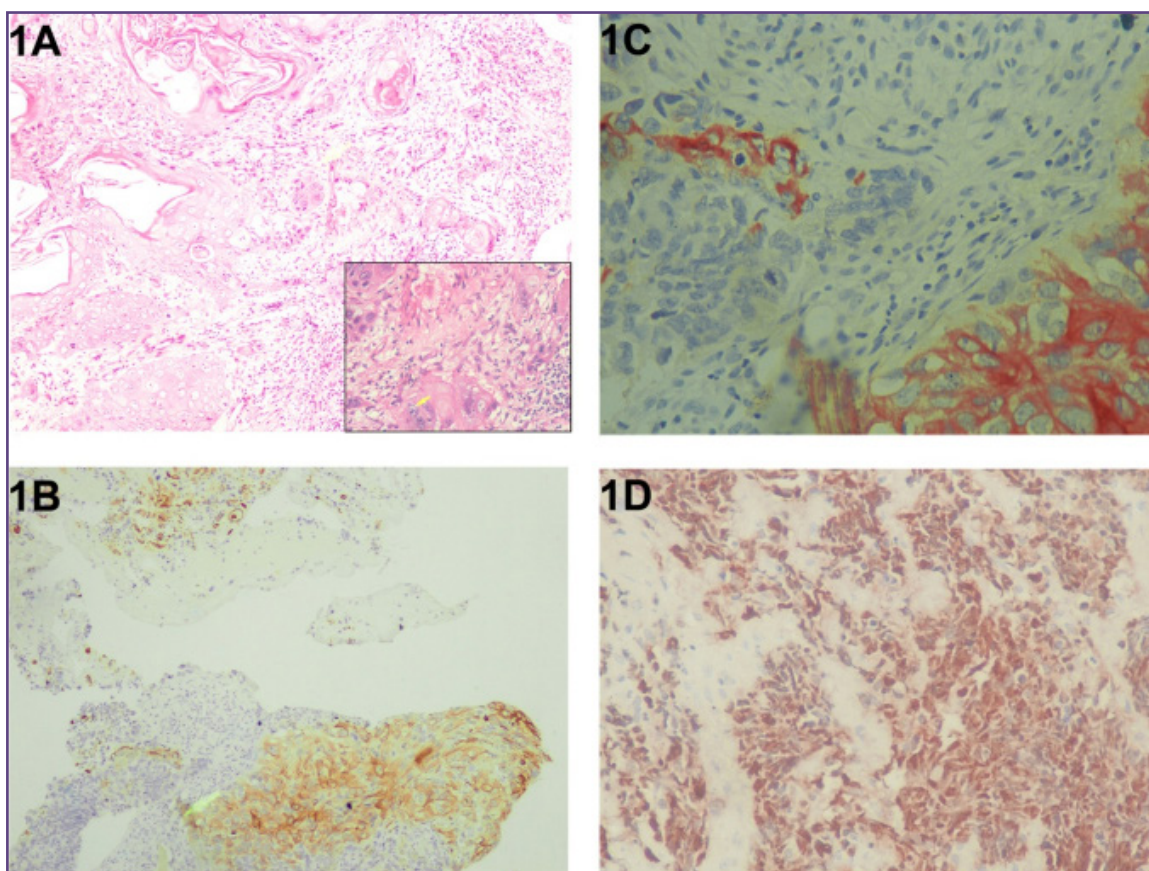
Fig. 1A : Squamous cell carcinoma- Showing tumour cells arranged in sheets and nests (H&E stain, 100x) Inset: Showing polygonal tumour cells , having hyperchromatic nuclei & prominent nucleoli (H & E stain , 400x); 1B : Squamous cell carcinoma- Neoplastic cells showing focal cytoplasmic positivity. (immunostain CK5/6, 100x); 1C: Squamous cell carcinoma - Neoplastic cells showing focal cytoplasmic positivity (immunostain CK5/6 ,400x); 1D : Squamous cell carcinoma - Neoplastic cells showing strong nuclear positivity (immunostain Ki-67, 400x).
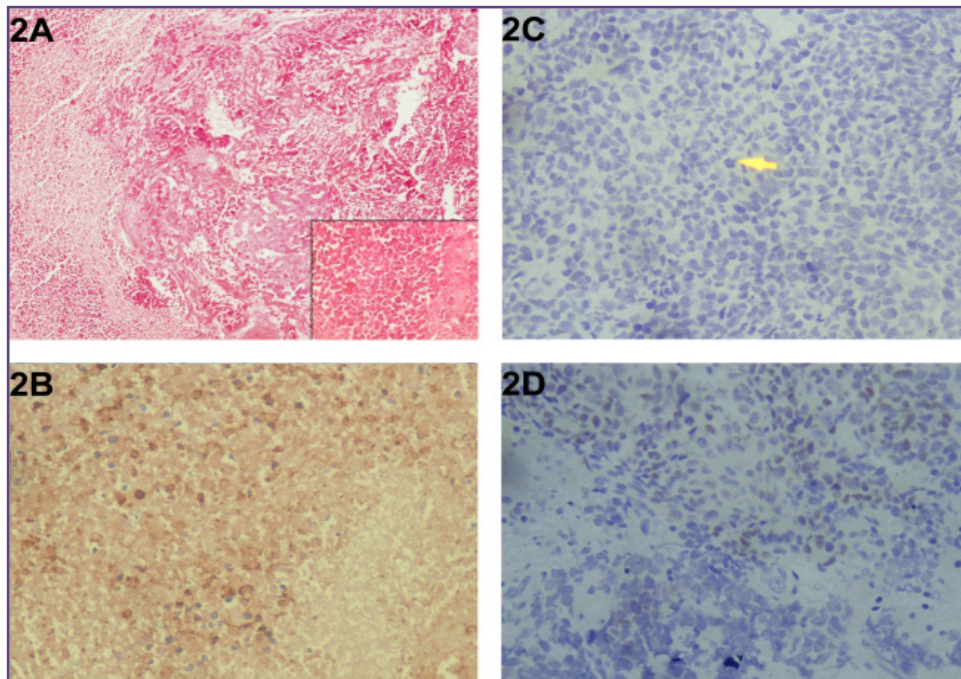
Fig. 2A : Poorly differentiated carcinoma- Showing large areas of necrosis along with a tumour arranged in poorly cohesive sheet (H&E stain, 100x) Inset :Showing small to medium sized cells having round to irregular hyperchromatic nuclei (H&E stain, 400x); 2B : Poorly differentiated carcinoma - Neoplastic cells showing diffuse cytoplasmic positivity (immunostain CK20, 400x); 2C: Poorly differentiated carcinoma- Shows neoplastic cells negative for immunostain (CK5/6, 400x). Arrow-Atypical mitotic figure; 2D: Poorly differentiated carcinoma with ill formed neoplastic glands.

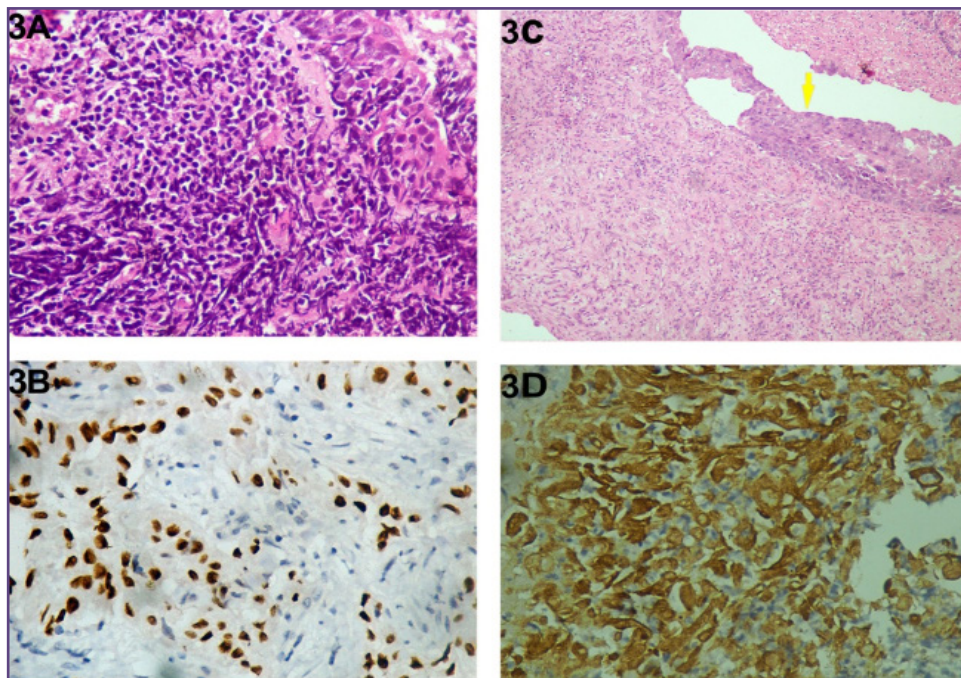**Fig. 3A : Small cell carcinoma- Showing small tumour cells having hyperchromatic nuclei and nuclear moulding(H&E stain, 100x); 3B: Small cell carcinoma - Neoplastic cells showing strong nuclear positivity (immnostain TTF-1, 400x); 3C: Suspicious of malignancy - Showing small foci of atypical cells (H&E stain 100x); 3D: Suspicious of malignancy- Neoplastic cells show strong diffuse cytoplasmic positivity for CK5/6 confirming it to be Squmous cell carcinoma (immunostain CK5/6, 400 x)**

non-squamous cell carcinoma from squamous cell carcinomas is neccessary for effective pulmonary cancer therapy specific for tumor subtype, and thus a number of studies are being undertaken by various institutions with this aim.[12,13] A combination of IHC panel is effective in reaching a diagnosis, however to date no consensus has been reached for the optimum panel of IHC marker to make a definitive diagnosis of lung carcinoma. Recent studies have advocated the combined use of antibodies for adenocarcinomas (TTF-1 and CK-7) and SCC (p63 and CK5/6) that provides a very reliable distinction between these 2 NSCLC subtypes.[14] Besides, some of the non-small cell lung carcinomas are too poorly differentiated to be accurately subclassified on routine H&E stain, especially if the biopsies are too small. However, recent immunohistochemical studies that focussed on poorly differentiated non-small cell carcinomas in small biopsies when combined with H&E serve as a gold standard for accurately subclassifying poorly differentiated non-small cell lung carcinomas.[15,16] Besides, TTF-1 and CK20 have ben recently reported to be useful to distinguish between primary and metastatic lung adenocarcinoma.

In this study, we intended to employ a minimum IHC panel that can be used in a tertiary care centre to reach a definite diagnosis in difficult cases. Our study employed the use of four antibodies namely CK5/6, TTF-1, CK20 and AE1/3. Of these CK5/6 and TTF-1 were use to differentiate between Squamous cell carcinoma and Adenocarcinoma in our study.

We observed out of 29 cases of Squamous cell carcinoma diagnosed on H&E, 26 cases(89.7%) were positive for CK5/6, thus confirming the diagnosis of SCC on IHC. Whereas, the 3 cases that were diagnosed as Squmous Cell carcinoma on H&E did not show a positive expression for CK5/6. However, all these cases were positive for AE1/3 which is a pancytokeratin. This erroneous staining pattern may be attributable to an increased crush artefact. All the 29 cases were negative for TTF-1. Thus, similar to other studies, CK5/6 is an excellent marker with certain limitations.[17,18]

In our study, out of 5 cases that were diagnosed as adenocarcinoma on H&E, 4 cases (80%) were positive for TTF-1, thus confirming primary Adenocarcinoma on IHC. One case that was negative for TTF-1, on employing CK 20 showed positive reaction thus confirming it to be Metastatic Adenocarcinoma. All these cases were negative for CK5/6.

TTF-1 is a marker for lung adenocarcinoma and is routinely used in diagnosis of pulmonary adenocarcinoma.[19,20] Furthermore, primary and metastatic Adenocarcinoma

of lung could be differentiated employing TTF-1 and CK20. In a study by Yue-Chi Su et al, 73% of primary lung adenocarcinomas expressed TTF-1 staining, whereas all non pumonary adenocarcinomas lacked TTF-1 staining. CK 20 expression was significantly more prevalent in metastatic adenocarcinomas.[16] They concluded that a combination panel could distinguish between pulmonary and extrapulmonary adenocarcinomas metastatic to lung.

It is interesting that use of IHC did not improve diagnostic accuracy for SCC in our study which is a finding similar to Ocque R et al .[21] In another study by Jorda et al, application of IHC markers increased the yield of detection of SCC from 35% to 88%.[22] The distinction in experiences with various studies may be attributable to the type of antibody used, amount of study material and experience of pathologist.

However, increased use of IHC markers as ancillary method was found to increase the interpretation and classification for adenocarcinomas as primary or metastatic. This is further supported by studies by other authors.[16-22] TTF-1 is a marker of lung adenocarcinoma and is routinely used in the diagnosis of pulmonary adenocarcinoma and this has been demonstrated in a number of studies.[23]

Out of 4 cases diagnosed as Small cell carcinoma on H&E, only 1 case (25%) could be confirmed to be Small cell carcinoma exhibiting both AE1/3 and TTF-1 positivity. The remaining 3 cases did not show a positive staining and may be attributable to small size of biopsy, crush artefact and observer variation. Our observation was in contrast to findings in a study by Miyauchi et al who observed TTF-1 immunoreactivity in 79 out of 96 cases (82%).[24] This may be attributable to small sample size of Small Cell carcinoma cases in our study.

Out of 4 cases diagnosed as Poorly differentiated carcinma on H&E, 3 cases could be further confirmed and diagnosed employing IHC markers. One case (25%) was confirmed to be Squamous cell Carcinoma exhibiting positive CK5/6 expression and other 2 cases exhibited positive CK20 expression (50%) thus confirming it to be metastatic adenocarcinoma. Thus the antibodies employed in present study were able to diagnose Poorly differentiated cases of lung carcinoma. Limited tumour size may be one of the factor for non- conformation of one case in our study.

In the category of Suspicious/Suggestive of malignancy on H&E included the cases that could not be definitely labelled as malignant due to small size of sample and features that fall short of malignancy. For 3 cases a confirmed diagnosis could be given on employing IHC markers. Of these 3 cases, 2 cases were confirmed to be

Squamous cell carcinoma exhibiting a positive expression for CK5/6 and one case exhibited a positive expression for TTF-1 thus confirming it to be primary adenocarcinoma. These 3 cases also exhibited a positive expression for AE1/3. One case that remained inconclusive even after applying IHC markers was asked for follow up and repeat biopsy. Thus, limitation of size of tumour poses difficulty in interpretation even on IHC.

The biopsies that showed epithelial dysplasia were grouped under the category of Others in our study and this group included 4 cases. However, positive expression of AE1/3 and CK5/6 was seen in only 1 case which confirmed it to be Squamous cell carcinoma. The remaining 3 cases in this category were negative for AE1/3, CK20, CK5/6 and TTF-1.

For squamous cell carcinoma, CK5/6 showed a sensitivity and specificiy of 100% and 57.1% respectively while TTF-1 showed a sensitivity and specificity of 100%and 96.7% respectively in our study. In a study by Kusum Kapila et al CK5/6 showed a sensitivity and specificity of 53% and 96% respectively and TTF-1 was found to be most specific marker for adenocarcinoma with 60% sensitivity and 98% specificity.[25]

Another study by Gregorz T Gurda et al in 72 cases of primary adencarcinoma TTF-1 showed a sensitivity and specificity of 84.5% and 96.4% respectively. In 30 cases of squamous cell carcinomaCK5/6 showed a sensitivity and specificity of 100% and 77.8% respectively[26]. Thus, our study is closely related with studies by other authors in India and West in respect to sensitivity and specificity of TTF-1 and CK5/6

Conclusion

In conclusion, our study showed that most of the lung tumours could be diagnosed morphologically if the biopsy is adequate. Use of IHC in poorly differentiated tumours help us to reach a definite diagnosis which is otherwise not possible on H&E.

After the development of widely available and applicable monoclonal antibodies, identification of immunophenotypic profile for most of the lung tumours is now possible. Definitive diagnosis on employing IHC is important for our clinical colleagues for better distinction between adenocarcinoma and SCC management.

Thus, using IHC either singly or a panel is a useful adjunct to morphological features and clinical parameters in the diagnosis and management of lung tumours. However, ancillary studies should not be used as additional diagnostic confirmation in which morphology itself is sufficient in subtyping of NSCLC.

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