Original Article

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Pathology Section

Cytohistopathological Association and the Use of a Dual Immunohistochemical Regimen in the Diagnosis of Lung Malignancies: A Cross-sectional Study

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ABSTRACT

Introduction: Lung cancer is a highly aggressive malignancy that causes significant morbidity and mortality. The incidence of lung cancer has been increasing in the past few decades. Cytology aids in the initial evaluation and diagnosis of patients with lung cancer. Currently, the classification of lung carcinoma has expanded beyond small cell lung carcinoma and Non-Small Cell Lung Carcinoma (NSCLC). Precise subtyping of poorly differentiated NSCLC into adenocarcinoma and Squamous Cell Carcinoma (SCC) has a direct impact on patient management and prognosis. The morphologic diagnosis forms the basis and is further supplemented by a panel of immunohistochemical markers. Immunohistochemistry (IHC) is important in cases with poorly differentiated morphology or partial sampling. The IHC panel used includes Tumour Protein p63 (p63) and Thyroid Transcription Factor (TTF1) for subtyping lung cancer.

Aim: The present study was conducted with the aim of studying the age and gender distribution, risk factors, cytohistopathological association, and formulating an effective IHC panel for the precise yet effective subtyping of poorly differentiated lung malignancies.

Materials and Methods: This cross-sectional study included cases retrieved from the Archives of Pathology Department, SRM Medical College and Research Centre, Chennai, Tamil Nadu, India, between July 2012 and July 2016. The cases included had a diagnosis of lung malignancy (confirmed by cytology/biopsy) or were suspected of having malignancy based on clinical/radiological findings. The study period was from July 2015 to August 2016. The cytology and biopsy slides were reviewed, and the malignancy was classified according to the World Health Organisation (WHO) classification of lung malignancies (2021). IHC was performed on the cases using the markers p63 and TTF1 as a dual regimen. Diagnosis and subtyping of tumours were done based on histomorphology, and the tumours were reclassified based on IHC findings. The data were statistically analysed using SPSS software version 25 and the ROC curve.

Results: The mean age of the patients was 60.9 years. The study included a total of 50 cases of lung carcinoma, with an average age of 60.9 years (ranging from 30 to 88 years). Among the cases, 35 (70%) had a positive smoking history. A concordant cytohistopathological association was observed in 26 (52%) of cases. Adenocarcinoma was the predominant subtype, accounting for 21 (42%) of cases. Tumour cells in adenocarcinoma showed positive staining for TTF-1, with the marker exhibiting 100% sensitivity and 83% specificity. In SCC, tumour cells were positive for p63, with the marker demonstrating 92% sensitivity and 82% specificity. Both markers showed effective sensitivity and specificity when used as a dual regimen.

Conclusion: Although lung cancer is typically diagnosed in the elderly population, there has been an increase in cases among younger individuals due to urbanisation. Smoking remains an important risk factor for lung malignancy. Exfoliative cytology alone is not sufficient for the diagnosis of lung malignancies and should be supplemented with biopsy for more accurate results. Adenocarcinoma was found to be the most common subtype in our study. The IHC panel of p63 and TTF-1 proved to be an effective regimen for classifying poorly differentiated lung carcinomas.

Keywords: Bronchial cytology, Lung carcinoma, Thyroid transcription factor 1

INTRODUCTION

Lung cancer is an aggressive malignancy that causes high morbidity and mortality. An increasing incidence of lung cancer has been observed worldwide in the last few decades [1]. Cytology plays a crucial role in the initial evaluation and diagnosis of patients. Currently, various sampling techniques are available for the cytologic evaluation of lung tumours, including abrasive, exfoliative, and Fine Needle Aspiration Cytology (FNAC) with image guidance [2]. NSCLC accounts for 80-85% of all lung carcinomas, with adenocarcinoma being the predominant histological type among NSCLC. Lung carcinoma classification has now expanded beyond small cell lung carcinoma and NSCLC, necessitating precise subtyping of NSCLC for effective treatment with targeted therapy based on the subtype. The histological classification has evolved to include molecular classification [3]. Most non-small cell tumours can be classified using a single adenocarcinoma marker (TTF-1 or mucin) and a single squamous marker (p63 or p40) [3].

Previous studies have shown that tumour cells in adenocarcinoma are positive for TTF-1, Napsin, and cytokeratin 7, while tumour cells in SCCs are positive for p63, cytokeratin 5/6, Neurotrophic Tyrosine Receptor Kinase (NTRK 1) , and NTRK 2 [4]. However, the challenge with these markers is that none of them individually provide tumour type sensitivity and specificity. Moreover, variable results have been reported over the years, necessitating further studies to evaluate the efficiency of each marker. Considering specimens with low cellularity, it is critical to use a minimal panel of IHC markers as it may be feasible to perform only a limited number of immunostains. Additionally, there is a growing need to conserve scarce material for predictive marker testing such as Epidermal Growth Factor Receptor (EGFR) and Anaplastic Lymphoma Kinase (ALK). Therefore,

an effective cocktail regimen of IHC markers is needed for the diagnosis of lung malignancies.

Recent studies have suggested the existence of distinct molecular pathways in the carcinogenesis of lung adenocarcinomas [4]. There are two distinct molecular pathways associated with lung adenocarcinoma carcinogenesis. One pathway is associated with smoking and activation of the K-ras oncogene, while the other pathway involves the activation of EGFR. EGFR mutations and ALK rearrangements are effective targets for EGFR tyrosine kinase inhibitors or ALK inhibitors in patients with advanced lung adenocarcinoma. These new molecular targets and driver mutations play a major role in pathogenesis and exhibit a significant response to therapeutic interventions.

With the knowledge mentioned above, the present study aimed to investigate the age and gender distribution, associated risk factors, cyto-histological association, and formulate an effective IHC regimen for the precise yet effective subtyping of poorly differentiated lung malignancies.

MATERIALS AND METHODS

It was a cross-sectional study in which cases were collected from July 2012 to July 2016 from the Archives of the Pathology Department at SRM Medical College and Research Centre. The cases included patients with a diagnosis of lung malignancy confirmed by cytology or biopsy, as well as cases with clinical or radiological suspicion of malignancy. Detailed medical histories were recorded from the case sheets, and the study was conducted with the approval of the Institutional Ethics Committee (IEC NUMBER: 818/IEC/2015).

Inclusion criteria:

- Cases clinically or radiologically suspected of lung malignancy.
- Bronchial cytology positive for malignancy.
- Lung biopsies or resected lung specimens positive for malignancy.

Exclusion criteria: Cases with primary tumours (clinically or radiologically proven) in sites other than the lung were excluded from this study.

Study Procedure

Cytology samples were obtained using flexible fiber-optic bronchoscopy performed by a pulmonologist. The slides were fixed in ethyl alcohol and stained with Haematoxylin and Eosin (H&E), Papanicolaou, and Giemsa stains. The cytology and biopsy slides were reviewed, and the malignancies were classified according to the WHO classification of lung malignancies (2021) [3]. Immunohistochemistry (IHC) was performed on sections from paraffin blocks using p63 and TTF1 markers [5]. IHC was conducted using the peroxidase-antiperoxidase method, and the primary antibodies for TTF1 and p63 were used, diluted in Phosphate-Buffered Saline (PBS).

During IHC, cases were considered positive if nuclear staining was present in atleast 10% of the tumour cells [6]. These criteria were applied to both TTF1 and p63 markers. Diagnosis and subtyping of tumours were determined based on histomorphology and post-IHC analysis. The data were analysed to determine the cyto-histological association and the individual relationship of each IHC marker with the corresponding histopathology. Statistical analysis was performed using SPSS software version 25.0, and ROC curve analysis was conducted.

RESULTS

A total of 50 cases of lung carcinoma were studied. The age distribution ranged from 30 to 88 years, with a mean age of 60.9 years [Table/Fig-1]. Two cases were observed in patients below 35 years of age. The gender distribution was 7:1 (male to female ratio) [Table/Fig-1]. Among the cases in our study, 70% (35/50) had a history of smoking. Among smokers, the predominant subtype was SCC (15/35), while among non-smokers, the predominant subtype was adenocarcinoma (7/15) [Table/Fig-2].

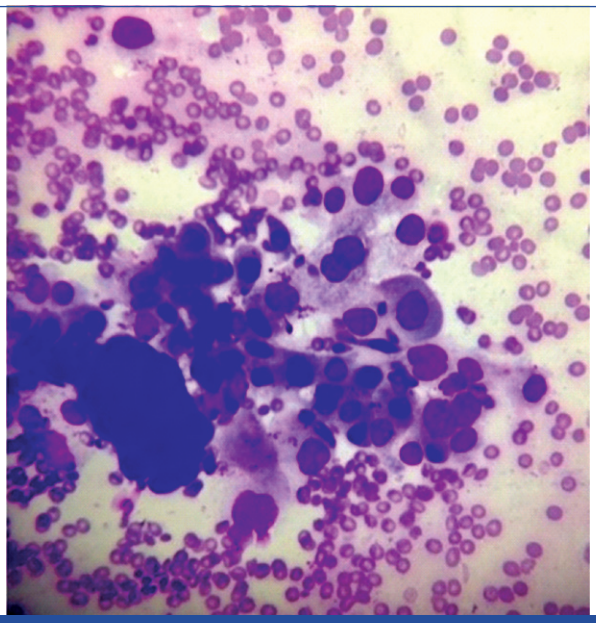
Bronchial cytology showed malignancy in 28/50 cases and was negative for malignancy in 22/50 cases [Table/Fig-3]. Out of the 50 cases, 48 were diagnosed as lung carcinoma on biopsy. Two cases showed positive results on bronchial cytology but had negative results on biopsy. A concordant cytohistological association was observed in 52% (26/50) of cases [Table/Fig-4]. Among the cases with negative bronchial cytology, biopsy predominantly showed adenocarcinoma (10/22) cases.

Age (in years)	Number of cases (n)	Percentage (%)
31-40	3	6
41-50	4	8
51-60	21	42
>60	22	44
Total	50	100
Gender		
Male	44	88
Female	6	12
Total	50	100

[Table/Fig-1]: Age distribution and gender distribution.

Parameters	Adenocarcinoma	Squamous cell carcinoma	Neuroendocrine	NSCLC-NOS	Adenosquamous	Large cell	Negative for malignancy	Total
Smoker	14	15	0	5	0	1	0	35
Non smoker	7	2	2	1	1	0	2	15
Total	21	17	2	6	1	1	2	50

[Table/Fig-2]: Association of smoking with the subtypes of lung carcinoma.
NSCLC-NOS: Non small cell lung carcinoma not otherwise specified

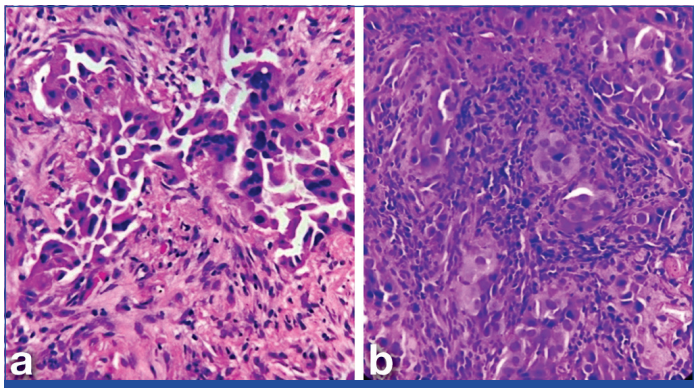


[Table/Fig-3]: Positive for malignancy-bronchial cytology (Haemotoxylin and Eosin, 400x).

Diagnosis by histomorphology revealed nearly equal cases of adenocarcinoma (17/50) [Table/Fig-5a] and SCC (15/50) [Table/Fig-5b], with 14 cases showing a poorly differentiated morphology [Table/Fig-6]. IHC was performed on the cases using a dual regimen

Diagnosis by bronchial cytology	Total	Final diagnosis on biopsy specimens (after IHC and review of H&E slides IHC plus H&E)						
		Adenocarcinoma	Squamous cell carcinoma	Neuroendocrine	NSCLC-NOS	Adenosquamous	Large cell	Negative for malignancy
Positive	28	11	11	2	1	1	0	2
Negative	22	10	6	0	5	0	1	0
Total	50	21	17	2	6	1	1	2

[Table/Fig-4]: Association of bronchial cytology results with subtypes of lung carcinoma.

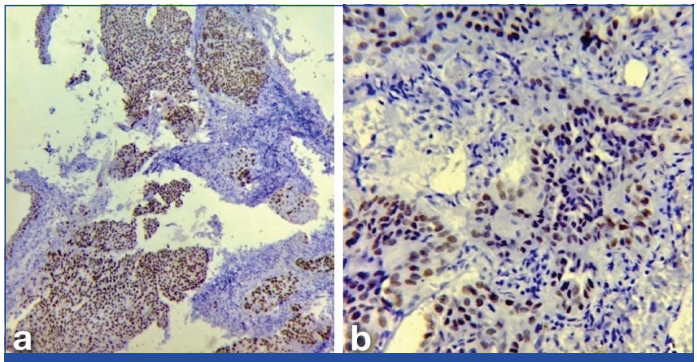


[Table/Fig-5]: a) Adenocarcinoma: Lung (Haemotoxylin and Eosin, 400x); b) Squamous cell carcinoma: lung (Haemotoxylin and Eosin, 400x).

Diagnosis	Number of cases (n)	Percentage (%)
Adenocarcinoma	17	34
Squamous cell carcinoma	15	30
Poorly differentiated	14	28
Neuroendocrine	2	4
Negative for malignancy	2	4
Total	50	100

[Table/Fig-6]: Diagnosis of lung cancer cases by Histomorphology (H&E).

of p63 and TTF-1. p63 positivity was observed in SCC [Table/Fig-7a], while TTF-1 positivity was observed in adenocarcinoma [Table/Fig-7b]. The cases were reviewed with IHC findings, and a final diagnosis was given [Table/Fig-8]. The predominant subtype in the present study (after IHC) was adenocarcinoma (21/50), followed by SCC (17/50).



[Table/Fig-7]: a) p63 positivity >10% tumour cells in squamous cell carcinoma-lung (100 x); b) TTF1 positivity >10% tumour cells in adenocarcinoma-lung (400x).

Seventeen cases of adenocarcinoma diagnosed based on histomorphology were confirmed to be the same after the IHC study, while 15 cases of SCC were modified to one case of adenocarcinoma, one case of adenosquamous carcinoma, one case of NSCLC-NOS (Not Otherwise Specified), and 12 cases remained as SCC. Among the 14 cases of poorly differentiated carcinoma, three were subtyped as adenocarcinoma, five as SCC, five as NSCLC-NOS, and one as large cell carcinoma. One case of adenosquamous carcinoma diagnosed in the present study showed positivity for both p63 and TTF-1. Six cases had a poorly differentiated morphology on H&E staining, and p63 and TTF-1 were negative, leading to a diagnosis of Non-Small Cell Lung Carcinoma-Not Otherwise Specified (NSCLC-NOS) after the IHC study [Table/Fig-9]. The total number of NSCLC-NOS cases was 6/50 (12%).

The p63 and TTF-1 showed good sensitivity and specificity results [Table/Fig-10], which were verified with ROC curve analysis [Table/Fig-11a,b]. The ROC curve for TTF-1 showed an area under the curve of 0.917, and the ROC curve for p63 showed an area under the curve of 0.873.

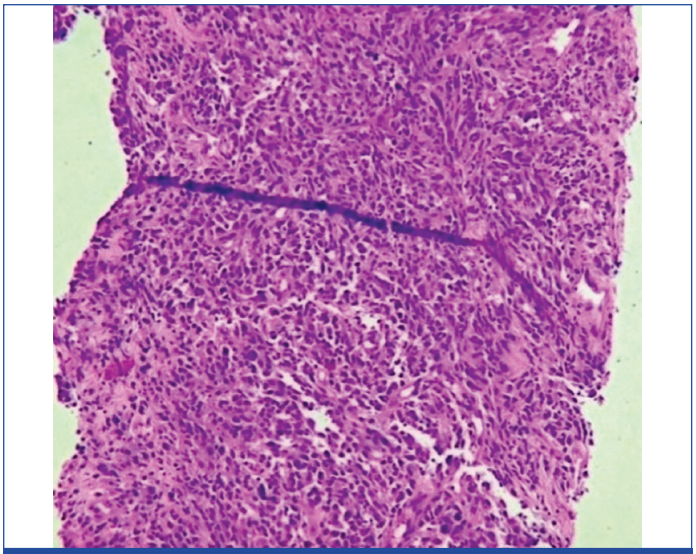
DISCUSSION

The incidence of lung cancer is increasing, leading to high mortality and morbidity rates. Early and accurate diagnosis, along with effective treatment, is crucial to improve the five year survival rate. In a developing country like India, the five year survival rate is only 5% [7]. Lung cancer exhibits significant genetic heterogeneity, requiring molecular characterisation for proper management. Surgery remains the primary treatment for Non-Small Cell Lung Cancer (NSCLC), particularly for stage I and II diseases [8]. Adjuvant chemotherapy may be beneficial for stage II tumours. Advanced-stage disease is typically treated with a combination of radiotherapy, chemotherapy, and targeted therapies. Small cell lung carcinoma is primarily managed with chemotherapy and radiotherapy, as surgery has limited efficacy. Therefore, accurate subtyping of lung carcinoma is essential for effective treatment and prognosis.

In the present study, the average age of patients was 60.9 years, with two cases observed in individuals below 35 years. The majority of cases were noted in patients over the age of 50, which is consistent with other studies [4,9]. The occurrence of lung cancer in younger individuals may be attributed to factors such as urbanisation and industrialisation, which increase the risk of the disease. Advanced diagnostic techniques and recent advancements enable early detection of carcinoma. The male-to-female ratio in our study was 7:1. The higher incidence of cancer in males can be attributed to factors like smoking and occupational exposure to hazardous

Pre IHC (Histomorphology)	Total	Post IHC (with review of histomorphology)						
		Adenocarcinoma	SCC	Neuroendocrine	NSCLC-NOS	Adenosquamous	Large cell	Negative for malignancy
Adenocarcinoma	17	17	0	0	0	0	0	0
Squamous cell carcinoma	15	1	12	0	1	1	0	0
Poorly differentiated	14	3	5	0	5	0	1	0
Neuroendocrine	2	0	0	2	0	0	0	0
Negative for malignancy	2	0	0	0	0	0	0	2
Total	50	21	17	2	6	1	1	2

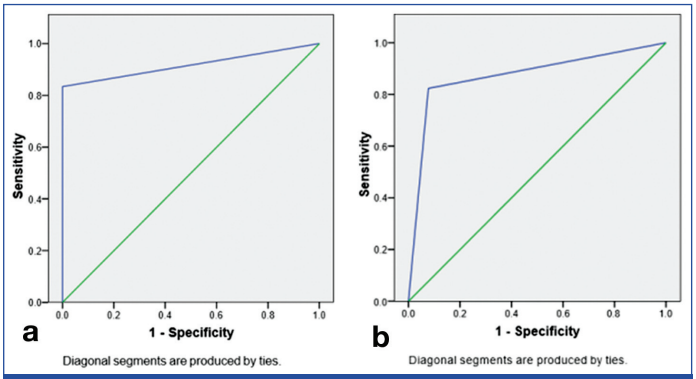
[Table/Fig-8]: Diagnosis of lung cancer cases with histomorphology and with post IHC review.



[Table/Fig-9]: NSCLS-NOS: Lung (Haemotoxylin and Eosin, 100x).

Parameters	TTF-1	p63
Sensitivity	100%	92%
Specificity	83%	82%
Positive predictive value	80%	80%
Negative predictive value	93.3%	93.3%

[Table/Fig-10]: Comparing sensitivity and specificity IHC markers with histopathology as gold standard.



[Table/Fig-11]: a) ROC curve for TTF1; b) ROC curve for p63.

agents, which are more common in males. Other studies have also reported a male preponderance, consistent with the findings [4,9].

Seventy percent of the cases in the study had a positive smoking history, highlighting its significance as a risk factor. Previous studies have shown a strong association between smoking and SCC [9]. Among the cases of SCC in the study, 88.2% were smokers and 11.8% were non-smokers. In contrast, adenocarcinoma was the most common type among non smokers. The present study revealed a concordant cyto-histological association in only 52.0% of cases. Negative cytology findings may be attributed to the peripheral location of adenocarcinomas, which can be missed during bronchoscopic procedures [10]. Additionally, the mucinous variant of adenocarcinoma may contain pools of mucin with few neoplastic cells, which can be overlooked in routine bronchoscopy-assisted cytologic studies [10]. Other cases with negative cytology results could be due to sampling errors. The accuracy of diagnosis depends on the experience of the cytologist, the cytologic method used, and the proper harvesting and processing of the pathological material. Previous studies have shown that biopsy provides better diagnostic yield compared to cytology, even in endoscopically visible carcinomas. FNAC, either endobronchial or transbronchial under image guidance such as ultrasound or Computed Tomography (CT)-guidance, is advisable for cytological procedures. Percutaneous or transthoracic approaches can also be used for FNAC. The false positive results on cytology (2/28) may be due to cytologic mimics that can mislead the cytopathologist [2]. In the present study, bronchial biopsy was used to validate the cytological techniques.

Subtyping of lung carcinomas was based on the algorithm followed by the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) International Multidisciplinary Team WHO classification 2021 [3]. Prior to IHC, the most common subtype based on morphology in our study was adenocarcinoma (17/50), followed by SCC (15/50), and 14/50 cases were reported as poorly differentiated carcinoma. In two cases, biopsy was negative but reported as positive on bronchial cytology. Two cases were reported as neuroendocrine tumours. False positive results in cytology may be due to cytologic mimics such as squamous metaplasia, reactive bronchial cells, or chemoradiation-induced changes [10]. The authors encountered a significant number (14/50) of poorly differentiated tumours, which could be due to less representative tissue or inadequate sampling. Tumour heterogeneity can also pose challenges in accurately assessing the histology of the tumour [11]. Poorly differentiated morphology is commonly observed in solid adenocarcinoma, non-keratinising SCC, or NSCLC-NOS, where typical adenocarcinoma or squamous features are not evident.

Histopathological diagnosis serves as the foundation and is complemented by a panel of IHC markers. In the study, IHC was performed using dual markers p63 and TTF1. The recent WHO classification of lung tumours (2021) emphasises the use of IHC in classification, particularly in small biopsies [3]. Adequacy of tumour tissue for molecular profiling is a critical concern, especially in lung cancer where small core biopsies limit tissue yield. To conserve tissue and prevent exhaustion, minimal yet effective IHC markers must be employed for subtyping [4]. Most non-small cell tumours can be classified using a single adenocarcinoma marker (TTF-1 or mucin) and a single squamous marker (p63 or p40).

The initial diagnosis was modified in certain cases following IHC and slide review. For example, one isolated case initially reported as SCC showed positivity for both TTF1 and p63. Upon review, a focal adenocarcinoma component (>10%) was identified. According to WHO criteria, it was reclassified as adenosquamous carcinoma. Adenosquamous carcinoma is a rare and highly aggressive lung malignancy [11]. Six cases were classified as NSCLC-NOS (non-small cell lung carcinoma, not otherwise specified) due to their poorly differentiated morphology and negative IHC staining for p63 and TTF-1. Sensitivity and specificity for the markers were calculated, and in the study, TTF1 was found to be 100% sensitive and 83% specific, while p63 was 92% sensitive and 82% specific. Adenocarcinoma was the predominant subtype in the study (21/50), consistent with other studies [4]. Six cases (12%) were categorised as NSCLC-NOS. The incidence of NSCLC-NOS is increasing, and these cases typically have a poor prognosis. Other important markers include CK5/6 or p40 for SCC, Napsin A for adenocarcinoma, and synaptophysin/chromogranin for neuroendocrine tumours [11].

In the future, the present study can be expanded with a larger sample size and a wider panel of IHC markers to enhance sensitivity and specificity. Additionally, molecular profiling of genes such as EGFR, ALK, and others implicated in lung cancer should be investigated to enable targeted therapeutic approaches for suitable patients.

Limitation(s)

A larger sample size is required to study the application of these findings in the population. A small sample size is a limitation of the study.

CONCLUSION(S)

The data of the present study confirmed the current epidemiological data regarding lung cancer, including age distribution, gender

distribution, and association with smoking. The results suggested that exfoliative bronchial cytology should be supplemented with histological diagnosis (endobronchial/transbronchial/CT-guided biopsy) for an effective diagnosis. Additionally, subtyping of poorly differentiated carcinomas using IHC markers p63 and TTF1 revealed that adenocarcinoma was the most common type, followed by SCC. The study highlighted the importance of reviewing the histomorphological features in conjunction with the IHC results to achieve an accurate and final diagnosis for specific treatment approaches.

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