Utility of Immunohistochemistry in the Subtyping of Lung Carcinoma on Small Biopsies

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ABSTRACT

Background: Hamstring tendon auto grafts in the form of quadrupled Background and objectives: In the era of precision medicine, the important task of today's pathologist is to classify lung carcinoma into specific histologic subtypes. Thus enables the scope to identify the molecular target for targeted therapy. Hematoxylin and eosin stain alone cannot subtype lung carcinoma when particularly it exhibits solid pattern of growth. This challenge becomes more pronounced when working with small biopsy samples.

Methodology and result: A retrospective cross-sectional study was conducted to evaluate small biopsies obtained by image guided lung core or bronchoscopy over three years, from 2020-2022. Tumour morphology was evaluated, and immunohistochemistry was performed in 132 cases of lung carcinoma. In this study a small panel of three markers (TTF-1, p63 and synaptophysin) was applied as an initial approach to classify different subtypes of lung carcinoma. Additional markers (such as NapsinA, p40, CK5/6, CD56, INSM1, CK7, CK20, etc.) were incorporated based on factors like morphological characteristics, clinical information, imaging data, and the results of the initial marker panel. The largest group observed in this study comprised 67% of adenocarcinomas that showed positivity for either TTF-1 or NapsinA. Squamous cell carcinomas, identified either through p63 expression or other markers such as p40/CK5/6, accounted for 26% of cases. Additionally, 10% of cases demonstrated reactivity to neuroendocrine markers, indicating the presence of neuroendocrine tumors. Notably, immunohistochemistry successfully identified the metastatic site for 10 adenocarcinomas that were negative for both TTF-1 and NapsinA.

Conclusion: A small panel of immunomarkers can classify the lung cancer reliably and increases the confidence at all level of lung cancer management.

Key words: *Non-small cell lung carcinoma, immunohistochemistry, Adenocarcinoma, squamous cell carcinoma, TTF-1, p63, p40*

BACKGROUND

Lung cancer is the leading cause of global cancer incidence and cancer related mortality with an estimated 1.8 million deaths[1]. Notably, lung cancer has emerged as the most frequently observed cancer in the Bangladeshi population, regardless of gender, as per institutional data[2,3]. In the era of precision medicine, accurately subtyping non-small cell lung carcinoma (NSCLC) holds significant importance aiming to provide the right drug to the right patient at the right time. The fifth edition (2021) of the World Health Organization (WHO) blue book on thoracic tumours emphasizes the integration of Immunohistochemistry (IHC) and genetic analysis with histopathological features to classify lung cancer[4]. IHC enables pathologist to precisely determine NSCLC subtypes using small panel of markers. From a practical standpoint, the selection of IHC panels were more inclusive of all possible scenarios of cancer pathology including the diagnosis of uncommon subtypes, and distinction of primary pulmonary from metastatic cancers. IHC proves particularly valuable in cases where histological features are ambiguous or when pathologists seek to enhance their confidence in the diagnosis.

METHODS AND MATERIALS

The study protocol was conducted at a histopathology laboratory within a tertiary care hospital in Dhaka between 2020 and 2022. A total of 346 image-guided lung core or bronchoscopic biopsies (Figure 1) were done during this period.

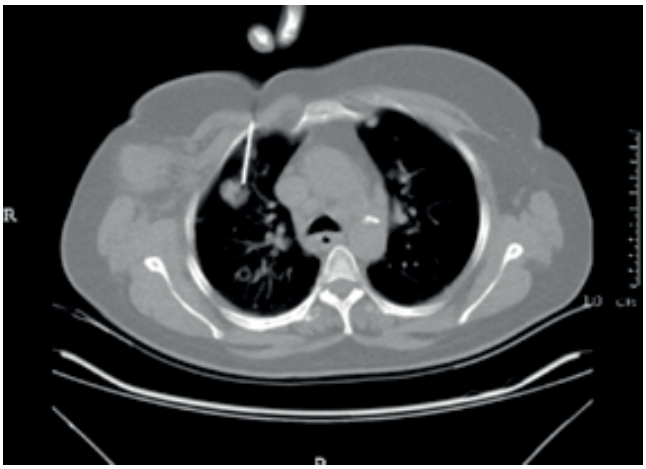

Figure 1: CT-guided Percutaneous core needle biopsy

It is to note that this study focuses solely on immunohistochemistry analysis of epithelial neoplastic lesions and does not encompass nonepithelial neoplastic lesions. The inclusion of lung cancer cases in this study was based on the requests from physicians for IHC analysis. Initially, a morphological evaluation of the tissue sections stained with Hematoxylin and Eosin was carried out to select the appropriate markers. As an initial panel, a small set of three markers (TTF-1, p63, and synaptophysin) were applied for subtyping lung carcinoma. Additional markers (such as Napsin A, CK5/6, p40, Chromogranin, CD56, CK7, etc.) were incorporated based on the morphology, clinical and imaging information, and the results of initial panel. The study adhered to an algorithm that was developed in alignment with the 2021 WHO classification of lung tumor (refer to Figure 2) for the interpretation of immunoreactivity[4-6].

The recommended guidelines for interpreting immunohistochemistry (IHC) results are as follows:
If a tumor is positive for either TTF-1 or Napsin A, regardless of p63, p40 or CK5/6 staining, it will be considered adenocarcinoma (ADC). On the other hand, if the tumor shows diffuse and strong positivity for one squamous marker (p63 or p40 or CK5/6) in the absence of TTF-1 and Napsin A expression, it will be classified as squamous cell carcinoma (SCC). The threshold for positivity varies for TTF-1 and p63/p40.

TTF-1 is considered positive when there is weak but definite staining present (>5%) in tumor cells. In contrast, for p63/p40 IHC, a high threshold is applied to determine the lineage of SCC; the majority (>50%) of nuclei need to exhibit strong positivity[7-9].

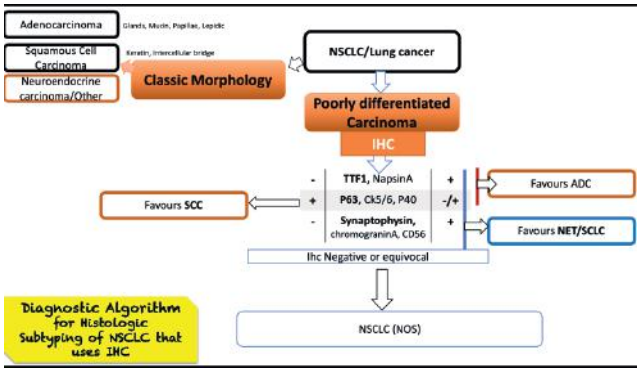

Figure 2: Immunohistological algorithm in subtyping of lung carcinoma

Abbreviation
NSCLC: Non-Small cell lung carcinoma, ADC: Adenocarcinoma, SCC: Squamous cell Carcinoma, NET: Neuroendocrine tumour, SCLC: Small cell lung carcinoma NSCLC(NOS): NSCLC (Not otherwise specified)

RESULT

A subset of cases were selected (figure 3) from a pool of 346 patients who underwent image-guided lung core or bronchoscopic biopsy. Among these cases, 240 were diagnosed epithelial cancer (carcinoma) through histopathological evaluation. Among these cases, IHC was performed in 132 (55%) cases (Figure 3). Patient characteristics of these 132 patients are shown in Table 1.

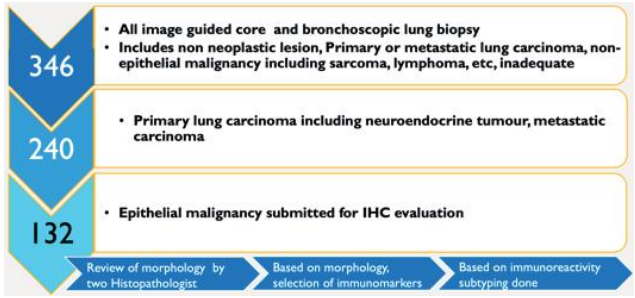

Figure 3: Selection of patient for IHC

Table 1: Patient characteristics:

Characteristics	No
Male/Female	99/33
M:F ratio	3:1
Age (years)	
Mean	65
Median	65
Range	41-82

Before applying Immunomarkers, histomorphology in H &E stain slides were reviewed. Out of 132 cases, 40 cases (30%) were with distinct histomorphology being diagnosed 29 as ADC, 8 as SCC and 3 as SCLC. Notably, Immunohistochemistry confirmed the morphological diagnoses for all these cases and additional information can be found in Table 2.

Table 2: Correlation of IHC findings with Carcinoma of differentiated morphology (40/132)

Histomorphology (No)	Immunomrker reactivity	Final Diagnosis
Adenocarcinoma (29)	TTF-1 (27/29), Napsin A (2/7)*, p63 (6/29)**	29/36
Squamous cell carcinoma (8)	p63 (6/8), p40 (2/8)*, CK5/6 (1/6)*	8/8
Small cell carcinoma (3)	Synaptophysin (3/3)	3/3

* Double markers used in some cases.
**Weak and heterogenous expression. Immunoreactivity showed 100% concordance with morphological diagnosis.

TTF-1 demonstrated robust nuclear immunostaining in adenocarcinoma (see Figure 4). p63 exhibited strong and homogeneous nuclear staining in SCC (Figure 4). Small cell carcinomas showed positive staining for NE markers (see Figure 4).

In the evaluation of total 132 cases of lung carcinoma, 70% (92/132) revealed diffuse, solid, nests, trabeculae, cords, single infiltrating cells or atypical epithelial morphology without gland formation or squamous differentiation. Assigning subtypes to tumors with such morphology, especially in small biopsies posed challenges, resulting in a classification of these cases as "NSCLC/Undifferentiated carcinoma." Initially, three primary immunomarkers (TTF-1, p63, and synaptophysin) were used to identify subtypes. Stepwise additional markers (Napsin A for adenocarcinoma, CK5/6 or p40 for squamous cell carcinoma) were employed when the initial markers showed non-reactivity or variable reactivity. Throughout the study, the range of immunomarkers were expanded to identify specific subtypes and metastatic lesions. Table 3 presents an overview of the immunomarkers used, aiding in the determination of lung carcinoma subtypes. Among the total of 132 cases, Immunohistochemistry identified 122 cases as primary lung carcinoma, while the remaining 10 cases were classified as metastatic carcinoma.

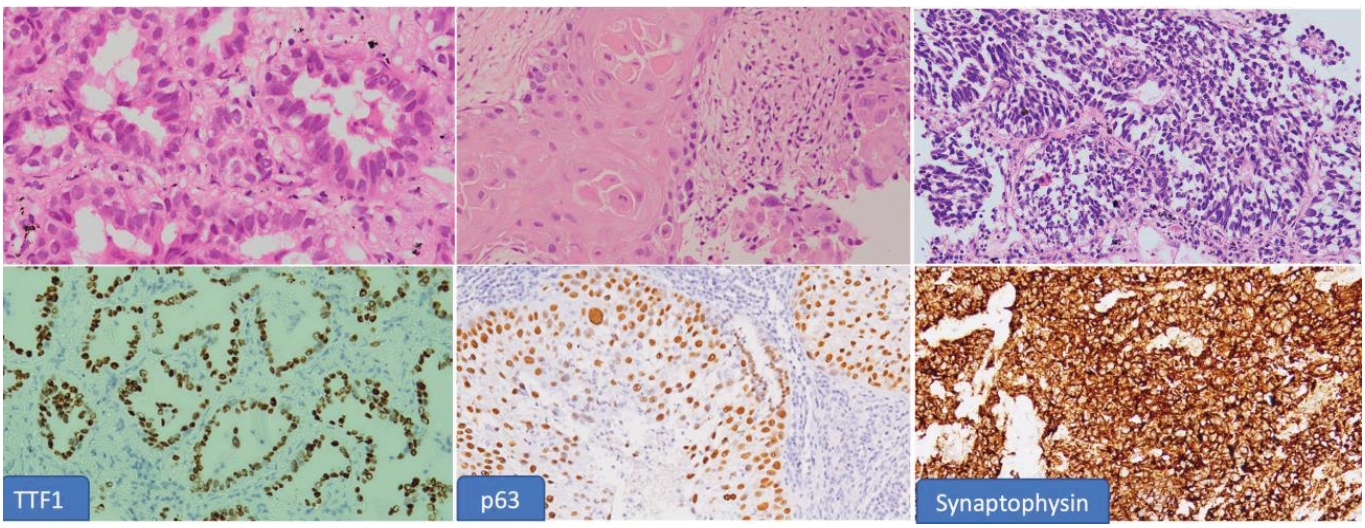

Figure 4: Tumour with differentiated morphology and immunoreactivity of corresponding markers substantiated the morphologic diagnosis.

Table 3: IHC profile of Lung carcinoma (n=122/132):

Immunoreactivity pattern (Reactivity and no employed)	Interpretation	Total (%)	Comment
Positive: TTF-1(69/74), Napsin A(8/18), p63(18/74), synaptophysin(10/74) Negative: p63 or squamous markers	ADC	74 (61%)	Double markers used in some cases.
Positive: P63 (23/27), p40 (6/20), CK5/6 (2/10) Negative: TTF1, NapsinA	SCC	27 (22%)	Double markers used in some cases
Synaptophysin (9/90), CD56, INSM1 (2/10), TTF1 (3/11)	NET	11 (9%)	9 SCLC, 1 LCNEC, 2 Carcinoid
Positive: p63, S100, SMA Negative: TTF-1	Other primary	5 (4%)	2 Adenosquamous, 3 Primary salivary salivary gland tumour
Positive: CK and CK7 Negative: TTF-1, Napsin A, p63, p40, CK5/6	NSCLS-NOS	5(4%)	Non-reactive to adenocrcinoma and squamous markers
CK7, CK20, CDX2, GATA3, PAX8, etc.	Metastatic ca	10	Confirmed with site specific immunomarkers

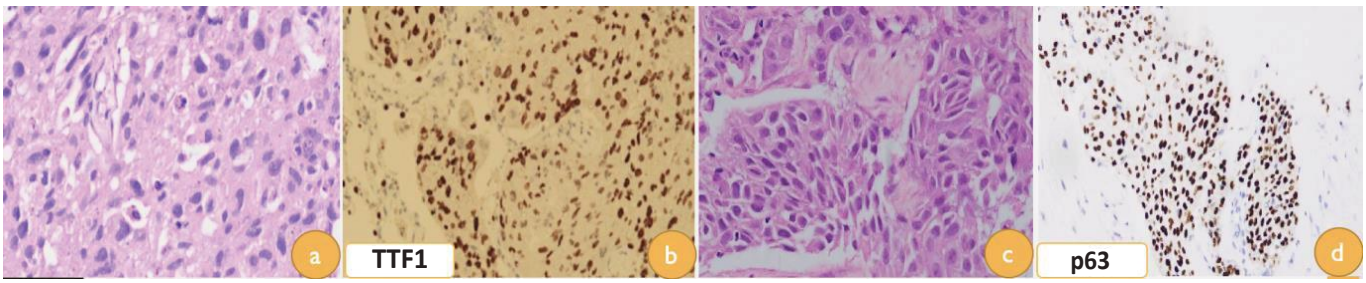

Figure 5: Non-small cell lung carcinoma -without any evidence of gland formation, mucin, squamous pearl formation or individual cell keratinization. (b) Nuclear TTF-1 positivity of (a) favors adenocarcinoma; (d) Nuclear p63 positivity of (c) favors squamous cell carcinoma.

Out of the 112 cases, which consisted of the three major histotypes (ADC, SCC, and SCLC), 101 cases (90%) were captured by the three markers (TTF-1, p63, synaptophysin). ADC, identified by either TTF1 or Napsin A positivity, was the most common subtype, accounting for 61% of primary lung carcinomas, while SCC accounted for 22%.

The sensitivity and specificity of TTF-1 and p63 to identify adenocarcinoma and squamous cell carcinoma of lung are shown in Table 4.

Table 4: Sensitivity and specificity of TTF-1 and p63 in relation to ADC and SCC

Markers	Reactivity	ADC N=74	SCC N=27
TTF-1	TTF-1 Positive	69 (TP)	0 (FP)
	TTF-1 negative	5 (FN)	27 (TN)
p63	p63 Positive	18 (FP)	24 (TP)
	P63 Negative	56 (TN)	03 (FN)

The sensitivity of TTF-1 was 93% to determine the adenocarcinoma.

In this study, all cases of mucinous carcinoma were subjected to additional markers (CK7, CK20, CDX2, STATB2, MUC) along with TTF-1/Napsin A. Among them, two cases showed negativity for both TTF-1 and Napsin A. One of which exhibited positivity for both CK7 and CK20 while other showed CK20+ and CDx2+ positivity. Despite extensive investigations, no primary site was discovered in any other part of the body including the gastrointestinal tract.

Neuroendocrine markers were positive in all NET including 9 small cell carcinomas as shown in Table 3. 27% of NET shows variable expression of TTF-1.

There were two cases of adenosquamous carcinoma where two separate cell population were distinct with immunostaining. One population showed immunoreactivity to adenocarcinoma markers (TTF-1+) and other population for squamous differentiation markers (p63+). Based on morphology and IHC features,

three salivary gland carcinomas were diagnosed as Adenoid cystic carcinoma, Epithelial-myoepithelial carcinoma and carcinoma ex-pleomorphic adenoma.

Five cases of non-small cell lung carcinoma with solid growth pattern could not be further classified even after IHC. Three cases only showed CK7 positivity and two cases only reactive to cytokeratin. Evaluation of clinical and imaging findings and additional immunostaining workup could not find other primaries.

Various IHC markers were employed to identify metastatic adenocarcinomas from cases of TTF-1/Napsin A negative adenocarcinoma. IHC picked up 12 metastatic adenocarcinoma along with their respective metastatic sites. Previous history of malignancy, clinical and imaging features substantiated IHC findings.

The initial diagnosis of adenocarcinoma of a TTF-1 negative but Napsin A positive had to be revised in this series. The confirmation of metastatic renal cell carcinoma (RCC) came from considering the patient's previous history of RCC and the reactivity to additional markers such as PAX8 and BCL2.

There is no primary pulmonary squamous cell carcinoma specific marker. p16 was added clinically ambiguous cases to rule out metastatic HPV induced SCC from cervix or Head and Neck. None of the SCC was p16 reactive in this series.

DISCUSSION

In this series, TTF-1 was a critical single marker in the decision-making tree. The minimalist IHC-based model utilizing two immunomarkers, one for adenocarcinoma and the other for squamous cell carcinoma, proved effective in this study. It could accurately subtype 90% of non-small cell lung carcinoma on small biopsy samples. The use of neuroendocrine markers may not be necessary in all cases of NSCLC or poorly differentiated carcinoma.

Two TTF1 staining offers two significant clinical advantages: distinguishing between lung adenocarcinoma from squamous cell carcinoma, and the differentiating the primary lung adenocarcinoma from non-pulmonary carcinoma[6]. The present study showed TTF-1 to be a highly sensitive and specific marker for lung ADC. Reported sensitivity of and specificity of TTF-1 ranging from 80 to 95% and 100% respectively for ADC [8,9]. Napsin A had been used in cases where TTF-1 is negative, TTF-1/p63 is negative or together with TTF-1 to identify missed cases of Adenocarcinoma. The limited utilization of Napsin A in this study prevented the evaluation of the superiority of one individual marker over others in lung cancer subtyping. Additionally, there was no opportunity to evaluate and contrast the performance of different clones same antibody. Various studies conducted on lung cancer have demonstrated comparable or superior sensitivity and specificity of Napsin A compared to TTF-1 in the diagnosis of adenocarcinoma[10,11]. In the current work, Napsin A expression was valuable when the TTF-1 expression results were inconclusive in ADCs. There were no cases of squamous cell carcinoma that tested positive either for TTF-1 or Napsin A. 33% Neuroendocrine tumour were TTF-1 immunoreactive.

p63 was the most used squamous cell marker in this series and its high sensitivity was worthwhile. This study also revealed weak and variable staining of p63 in 12% of adenocarcinoma. Despite this, our clinical practice has relied more on p63 than p40. Notably, In the literature p40 is preferred over p63 due to its comparable sensitivity and superior specificity[4,5,8]. However, in our clinical practice, we showed more dependence on p63 over p40. We need to align our practice with the WHO recommendation. In this series p40 and CK5/6 were used to find p63 negative squamous cell carcinoma.

Most used neuroendocrine marker in the study was synaptophysin. Besides Small cell carcinoma, there were 2 neuroendocrine tumour and one large cell neuroendocrine carcinoma detected. Other neuroendocrine markers used in these series were CD56, Chromogranin and INSM1. Synaptophysin shows variable expression in 10% ADC.

The immunoprofile of primary mucinous lung adenocarcinomas, colloid carcinomas, and adenocarcinomas with enteric differentiation differs from that of other adenocarcinomas and overlaps with gastric and pancreatobiliary tract carcinomas. These tumours often exhibit positivity for CK7, CK20, and CDX2 and can be negative for TTF-1 and Napsin A7.

There were instances of NSCLC where the results remained inconclusive despite the application of IHC markers (Negative for TTF-1 and p63/p40) labeled as NSCLC-NOS (Not otherwise specified). Tumour showed negative results for immunomarkers of primary lung carcinoma, a diagnostic panel comprising Pancyto

keratin, Vimentin. CD45 and S-100 protein was applied to fix the diagnosis of carcinoma and rule out mesenchymal, hematopoietic, and melanocytic neoplasms. Although IHC is a valuable technique, it is not without limitations. Technical issues such as suboptimal tissue processing, inadequate antigen retrieval, or variation in antibody sensitivity and specificity can lead to unreliable IHC results, making it challenging to assign a specific NSCLC subtype. To address this, the pathologist needed to be aware of the pitfalls of IHC, review the histomorphology, consider the clinical and radiological features of the patient, add supplementary markers, and repeat the immunostaining.

It is a challenge for a pathologist to accurately diagnose and classify lung cancer in small biopsy samples. Small biopsy specimens, which is the reality now, frequently have classification issues because of inadequate sampling or the existence of a very little quantity of tumors that might not exhibit differentiated characteristics.

Identifying NSCLC subtypes namely, adenocarcinoma and squamous cell carcinoma, is crucial for tailoring chemotherapy regimens and exploration for targetable molecular alterations such as EGFR, ALK, ROS1, PDL1, etc. in adenocarcinoma[12]. The rapid advancement in this field necessitates ongoing reassessment of existing practices, predictive testing, and widespread sharing of updated best practices.

CONCLUSION

To remove the uncertainty of diagnosis and accurate subtyping of lung cancer, immunohistochemistry has become the mandatory adjunct. When Tumor exhibits differentiated morphology, the use of IHC is unnecessary. The combination of one adenocarcinoma and other squamous markers seems adequate for reliably detecting the primary subtypes of lung carcinoma (NSCLC). Due to the diverse morphology of lung cancer, a wide range of markers is required to rule out other uncommon subtypes of epithelial and non-epithelial malignancies and distinguish between primary and metastatic tumors. The correlation clinical and radiological finding can minimize the excessive use immunomarkers and associated costs. When diagnosing small biopsies, it is crucial to carefully consider the efficient utilization of IHC to preserve tissue for molecular testing purposes.

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