Minimalistic Immunomarker Approach for the Diagnosis of Non Small Cell Lung Carcinoma with Emphasis on Tumours with Ambiguous Morphology

FADIYA ZAINUDEEN¹, PS JAYALAKSHMY², ASIQ SIDEEQUE¹

ABSTRACT

Introduction: Diagnosing Non Small Cell Lung Carcinoma (NSCLC) by morphology on small biopsy specimens are on demand. With major advances in molecular testing of lung cancers and introduction of targeted therapies, accurate subtyping of NSCLC has become essential. Immunophenotyping with various immunomarkers is helpful in identification of different tumour subtypes. As sparing material for molecular testing is mandatory, a minimalistic Immunohistochemistry (IHC) based diagnostic approach is warranted by means of reliable and accessible immunomarkers. In this study, one immunomarker Thyroid Transcription Factor (TTF1) for glandular and protein40 (p40) for squamous differentiation was applied to maximise the proportion of accurately subtyped NSCLC on small biopsy specimens.

Aim: To find utility of TTF1/p40 minimalistic Immunopanel in subtyping NSCLC especially those with ambiguous morphology.

Materials and Methods: This retrospective observational study was conducted from 15 December 2020 to 5 March 2021 in a tertiary care hospital in which 93 (mean age 64.8 years, Male:female ratio 4.8:1) consecutive lung biopsies from suspected lung carcinoma obtained from January 2019 to December 2020 were studied.

Results: The morphological diagnosis and diagnosis after application of TTF1 and p40 minimalistic immunopanel was evaluated using chi-square test. The p-value <0.01 was considered significant.

The objective of the study was to evaluate the utility of two marker approach p40 and TTF1 might help in subtyping NSCLC especially those with ambiguous morphology and reduction of category of NSCLC NOS significantly and contribute in sparing material for molecular testing.

INTRODUCTION

Lung cancer remains the most lethal cancer and also as the leading cause of mortality of all cancers worldwide [1]. Primary lung carcinomas are classified into Small Cell Lung Carcinoma (SCLC) and NSCLC, which accounts for 80% of all primary lung tumours [2]. The developments in targeted therapy against specific molecular alterations have necessitated precise subclassification of NSCLC which comprise ADC and SCC predominantly [3,4]. The reproducibility rate of the pathologic diagnoses, based on the morphological features is satisfactory for distinguishing SCLC from NSCLC and for identifying glandular versus squamous differentiation in low grade or well differentiated ADC and SCC. However, it is less satisfactory for subtyping high grade NSCLC cases with ambiguous morphology especially in tiny tissues [5,6]. Tumours with ambiguous morphology include poorly differentiated solid variant of ADC, poorly differentiated SCC with no squamous pearls or intercellular bridge, pseudoglandular variant of SCC, poorly differentiated ADC with metaplastic keratinising cells and large cell carcinomas.

The relationship of therapies and predictive biomarkers has made the handling of small biopsy specimens key factor for theranostic purposes [7,8]. The emerging evidence for differential responses of NSCLC specific subtypes to the new targeted therapies highlights the increasing need for a greater precision in such subtyping [6]. Several recent studies have addressed this issue, using different IHC panels to correctly distinguish lung ADC from SCC which constitutes majority of NSCLC [1,5,6,9].

Furthermore, a daily challenge in clinical practice involves how to use best out of minimal tumour tissue while making an accurate and rapid diagnosis. A paradigm shift of the traditional morphology related approach on small cytology and/or biopsy specimens has been recently incorporated into the World Health Organisation (WHO) 2015 classification of lung tumours which recommends using IHC and/or multidisciplinary setting to minimise the category NSCLC-NOS and sparing diagnostic material for predictive molecular testing [10,11]. Hence, a minimalistic IHC panel approach based on informative, reliable, reproducible, and easy-to-assess biomarkers is advisable for NSCLC to be typed for the sake of clinical usefulness and tenability of costs especially in resource poor countries [12].

Only few studies implicate the use of a limited panel of immunohistochemical markers comprising one marker of glandular differentiation such as TTF1 and one marker of squamous differentiation such as p40 [12,13]. p40 is an isof orm of p63 and denotes the non transactivating domain (deltaNp63) [14,15]. p40 has been shown to be superior to the commonly used antibody p63 (clone 4A4) for squamous differentiation as p63 may be found in some lung ADCs and even large cell lymphomas, making it less specific [15]. The objective of the study was to evaluate the utility of two marker approach p40 for SCC and TTF1 for ADC for accurate characterisation of NSCLCs, especially those with ambiguous morphology.
MATERIALS AND METHODS
This retrospective study was conducted in Department of Pathology, MES Medical College, Perinthalmanna, Kerala and included a series of 93 consecutive lung biopsies obtained during January 2019 to December 2020 for a period of 24 months from patients of suspected primary lung cancer attending Respiratory Medicine Department. The study period was from 15 December 2020 to 5 March 2021. Of all biopsy specimens, 24 were Computed Tomography (CT) guided biopsies and 69 were endobronchial biopsies. The study was approved by Institutional Scientific and Ethical Committee (Ethical clearance no.ECR/788/Inst/KL/2015-RR-18).

Inclusion criteria: All lung biopsy specimens obtained from Respiratory Medicine Department including Computed Tomography (CT)-guided biopsies and endobronchial biopsies in suspected primary lung cancer which was classified morphologically or immunophenotypically into NSCLC and its subtypes including ADC SCC or adenosquamous carcinoma were included in this study.

Exclusion criteria: Inadequate material on biopsy for IHC analysis. Morphological or immunophenotypic diagnosis of small cell carcinoma, large cell neuroendocrine carcinoma, others neuroendocrine tumour or mesothelioma. Patients having clinical or radiological evidence of secondaries in lung were excluded in this study.

Study Procedure
All biopsies were obtained in neutral buffered formalin, processed, embedded in paraffin and 4 microns sections were cut. Staining was done by standard Haematoxylin and Eosin (H&E) method and extra sections were cut on adhesive coated slides for IHC to prevent loss of tissue due to repeated trimming of the block. All study cases were annotated with available information in a way that protected patient privacy.

Morphological Examination
All NSCLC were diagnosed as ADC when there is definite acinar, tubular, lepidic, papillary, micropapillary patterns and/or intracytoplasmic mucin. SCC were diagnosed when characteristic intercellular bridges, keratinisation and keratin pearls are present. All cases with solid growth pattern or lacking any definite differentiation were grouped as NSCLC with ambiguous morphology.

Immunohistochemistry (IHC)
A IHC for p40 and TTF1 was performed on all biopsy specimens which were categorised into morphologically identifiable NSCLC either ADC or SCC and on National Comprehensive Cancer Network (NCCN) category with ambiguous morphology. Based on WHO 2015 and NCCN guidelines 2019, the cases were designated as NSCLC favour ADC, if immune profile was TTF1+/p40- and NSCLC favour SCC, if TTF1-/p40+, NSCLC-NOS, if TTF1-/p40- [11].

Methods: TTF1, thyroid transcription factor 1 (Rabbit anti-human TTF1 monoclonal antibody, clone EP229 from PathnSitu, Selbylane, Livermore, USA) in prediluted ready to use form and p40 (Rabbit anti-human p40 polyclonal antibody from PathnSitu, Selbylane, Livermore, USA) in prediluted ready to use form were used. Four micrometre sections were cut from tissue block, placed on pre-heated glass slides and kept at 60°C for one hour which was then dewaxed and rehydrated with graded alcohols. Antigen retrieval was done by heat induced epitope retrieval method. Blocking of endogenous peroxidase done with 3% hydrogen peroxide. Sections were reacted at room temperature with primary antibody for 30 minutes. Slides were then incubated in a detection kit (PolyExcel Horseradish Peroxidase/Diaminobenzidine (HRP/DAB) Detection System) according to manufacturer's instructions, developing peroxidase activity with 3,3’-diaminobenzidine tetrahydrochloride. Finally, slides were counter stained with haematoxylin, dehydrated and mounted.

Evaluation of immunostaining: Positive control tissue was used on each slide. Immune reactivity was rendered semi-quantitatively on scale from 0 to 3+ which was observed as follows. percentage of positivity was graded from 0 (absent staining), 1 (1-24%), 2 (25-49%) and 3 (50-100%) of tumour cells. Intensity was graded as 0 (no staining), 1 (weak), 2 (moderate) and 3 (strong). Score was calculated on a scale of 0 to 9 where 0-1=0, 2-4=1+, 5-7=2+, 8-9=3+ [13]. Morphological diagnosis and IHC analysis were performed independently by two pathologists at different time.

STATISTICAL ANALYSIS
Data was entered in Microsoft (MS) Excel and analysed using Statistical Package for Social Sciences (SPSS) software programme version 20.0 (International Business Machines (IBM) Corp., Armonk, NY, USA). Results were presented in terms of tables. The descriptive statistical percentages were calculated. Chi-square test was performed to assess the significance of association on the diagnosis of NSCLC before and after addition of IHC. The p-value of <0.01 was considered significant. Sensitivity and specificity of TTF1 and p40 were calculated. In adherence to fourth edition of the WHO 2015 classification of lung tumours, a case was rendered true positive for TTF1, if at least 1% of tumour cells were positive, irrespective of p40 immunostatus [11,16,17]. True positive for p40 were all cases showing p40 positivity and TTF1 negativity [11].

RESULTS
The median age of patients was 64.8 years. Male to female ratio was 4.8:1. ADC was most common cancer type in males and females. An 81.2 % (13/16) of tumours were ADC in females and it constituted 43 (55.8%) out of 77 of all tumours in males. Males had higher percentage of SCC and NSCLC-NOS compared to females. Based on morphological analysis, 21 (22.5%) cases out of 93 were morphologically subtyped into ADC and SCC. A 15 cases out of 21 were classified as ADC which showed definite glandular differentiation or intracellular mucin and six cases could be classified confidently as SCC which showed presence of intercellular bridges, keratin pearl and/or cellular keratin. Remaining 72 (77.4%) cases could not be further subtyped which showed ambiguous morphology with solid pattern and no definite differentiation, were grouped as NSCLC with ambiguous morphology.

Adenocarcinoma (ADC): Fifteen cases of morphologically identified ADC showed definite glandular differentiation [Table/Fig-1]. An 11 cases showed tubule formation, two cases lepidic pattern, one cribriform, one papillary and two cases showed occasional signet ring cells. Most cases showed mixed pattern. A three out of 15 cases were invasive mucinous ADC with glandular differentiation which showed tall mucin secreting cells and extravasated mucin.
Patterns identified were tubular, papillary and lepidic. Nuclei were hyperchromatic. One case showed haemorrhage and necrosis. The cases which showed mucin were confirmed with appropriate special stains.

**Squamous Cell Carcinoma (SCC):** Only six cases were showing definite squamous differentiation [Table/Fig-2]. Four cases showed nest and sheet pattern with intercellular bridges. Five cases showed keratin pearl formation and intracytoplasmic keratin. Four cases showed predominant hyperchromatic nuclei over vesicular type. Atypical mitotic figures were noted in two cases and one case showed apoptotic debris.

![Image](a.png)  ![Image](b.png)  ![Image](c.png)  ![Image](d.png)

**[Table/Fig-2]:** (a&b) Morphologically differentiated squamous cell carcinoma; (c) Strong diffuse p40 nuclear positivity (3+); (d) TTF1 negativity (H&E, 200X).

A 72 cases of NSCLC showed ambiguous morphology with no definite differentiation and could not be confidently subtyped. Most prevalent patterns were diffuse and solid which was shown by 42 cases. Patterns like nests, vague glands, trabeculae, cords and single infiltrating cells were also seen. A 52 cases showed predominant hyperchromatic nuclei. Only 20 cases showed vesicular nuclei and 28 cases showed prominent nucleoli. Necrosis was noted in 20 cases. A few cases showed apoptotic debris, atypical mitotic figures and intranuclear inclusions.

**Immunohistochemical analysis:** TTF1/p40 immunopanel was applied to two categories, one of which is morphologically evident subtypes of NSCLC that is ADC/SCC and to other category of the NSCLC with ambiguous morphology. Out of 93, 21 cases which were morphologically evident, 15 were ADC and six were SCC [Table/Fig-1,2]. In ADC, 10/15 showed TTF1+/p40-, 1/15 showed TTF1+/p40+, 1/15 showed TTF1-/p40- and 3/15 were invasive mucinous ADC which showed TTF1-/p40-.

When the panel was applied to SCC, all cases 6/21 showed TTF1-/p40+ [Table/Fig-3,4].

TTF1/p40 panel was applied to NSCLC-NOS category with ambiguous morphology which constituted 72 cases. A 36 out of 72 cases showed TTF1+/p40- and 3/72 showed TTF1+/p40+ which was classified as NSCLC favour ADC according to WHO 2015 and NCCN guidelines [11,16]. A 23/72 cases showed TTF1-/p40- which remained as NSCLC-NOS and could not be categorised further [Table/Fig-3,4].

Thus, altogether the panel succeeded in categorising 79 out of 93 cases into either ADC or SCC. A 50 out of 79 cases were classified as ADC, of which 46 out of 50 cases showed TTF1+/p40- and 4/50 showed TTF1+/p40+.

In ADC with TTF1 positivity, 2+ or 3+ positivity (moderate to strong intensity in >50% of cells) were noted. A 29 out of 79 cases were categorised as SCC based on TTF1-/p40+ immunopanel. All cases of SCC showed strong and diffuse positivity (3+) with p40. There were no cases of adenosquamous carcinoma in this study, for which TTF1 and p40 should stain different groups of tumour cells. Thus, there is significant reduction in NSCLC ambiguous category and significant increment in diagnosis of ADC and SCC subtypes compared to diagnosis based on morphological basis alone with...
application of TTF1/p40 minimalistic immunopanel with a p-value of <0.001 (chi-square test) as shown in Table/Fig-7.

<table>
<thead>
<tr>
<th>Morphology (%)</th>
<th>Addition of IHC (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSCLC-NOS (Ambiguous morphology)</td>
<td>72</td>
<td>10</td>
</tr>
<tr>
<td>Subtypes (ADC, SCC)</td>
<td>21</td>
<td>73</td>
</tr>
</tbody>
</table>

Sensitivity and specificity of immunomarkers: True positive cases with TTF1 were 50 and only four cases showed false negativity. No case showed false positivity with TTF1. Thus, sensitivity of TTF1 was 92.59% and specificity was 100%. Since all the cases of SCC showed strong diffuse positivity for p40 and no false negativity, sensitivity of p40 was 100%. But p40 showed 1+ (weak positivity in <25% cells) in four cases of ADC. Hence, false positivity was four and true negative cases were 50 resulting in specificity of 92.6%.

DISCUSSION
Recent advances in targeted therapy and chemotherapy of lung tumours require an accurate subclassification of NSCLC. NSCLC consisting tumours other than SCLC, accounts for majority of all lung tumours; lung ADC and SCC are most common histological subtypes [11,18]. According to WHO 2015 classification of tumours of lung, there is a shift in most common histologic subtype from SCC to ADC in recent years [11]. In present study, ADC is the most common lung carcinoma which is also the most frequent cancer in both genders. Mean age of occurrence is 64.8 years.

Morphologically, ADC is identified by glandular differentiation and mucin production. SCC is recognised by keratinisation, keratin pearls and intercellular bridges. But diagnosis of poorly differentiated tumour with ambiguous morphology is challenging. So, morphology alone is not very sensitive to accurately classify NSCLC into SCC or ADC on small biopsy specimens. The error rate in classifying NSCLC into appropriate histological subtype is high in small biopsies because it may not be representative of total tumour due to histological heterogeneity and distinguishable morphologic features of SCC and ADC differentiation are only identifiable if solid tumour component was sampled [10].

Many previous studies have demonstrated the utility of different immunomarker panels consisting of various markers such as TTF1, napsin A, Cytokeratin (CK7) for ADC and p63, CK5/6, CK34bE12, desmoglein-3 or desmocollin for SCC for subtyping of NSCLC [4,9,6,19]. In present study, TTF1 showed high sensitivity and specificity of 92% and 98% percent respectively, in diagnosing ADC. Sensitivity and specificity of TTF1 in primary ADC was 84.5% and 94.4% in a study by Gurda GT et al., [20]. In a two marker study, Walla R et al., obtained sensitivity of 85.3% and specificity of 100% in ADC diagnosis [13]. Muhammad FR et al., obtained sensitivity of 80.9% and specificity of 100% for TTF1 in ADC [21]. In a study, comparing clones of TTF1 on resected primary lung ADC and ADC metastasis to lung, which comprised of three different clones namely 8G7G3/1, SPT24, and SP141, found 8G7G3/1 clone to be more specific but less sensitive compared to SPT24 and SP141 [22]. But the clone used in this study is EP229 which was not included in that study. Another material sparing study by Guo R et al., utilised the technique of dual marker staining with CK5/6/TTF1 and p40/napsin A which showed high sensitivity and specificity and was comparable to single marker staining [23].

In current study, all morphologically classified SCC were positive for p40 and all of p40 positive NSCLC with ambiguous morphology were TTF1 negative making p40 100% sensitive. Based on literature review, a case with p40 negativity is less likely to be SCC [24]. Tacha D et al., described six cases of poorly differentiated lung SCC which were p40 negative, subsequent staining with other squamous markers were also negative [25]. Kadota K et al., re-classified 31 cases of lung tumours that were originally diagnosed as SCC into other histologic subtypes based on p40 negativity [26]. Rekhtman N et al., re-classified three tumours with initial SCC diagnosis and lack of p40 expression as solid ADC [27]. Affandi KA et al., reclassified eight cases of SCC into poorly differentiated or solid type ADC based on p40 negativity [24]. Present study showed slightly lower specificity of 92.6%. This may be attributed to the type of p40 clone used that is RP40 (rabbit polyclonal) which has shown to stain alveolar macrophages and non-specific cytoplasmic staining compared to MMp40 BC28 (mouse monoclonal) p40 clone which showed clear nuclear staining without non specific background stain. Affandi KA et al., used MMp40 clone and observed distinctive nuclear staining with clean background [24].

Current study was conducted to assess the validity of a minimalistic IHC panel comprising TTF1 and p40 in subtyping of NSCLC. Frequency of correct diagnosis of NSCLC on small biopsies by light microscopy varies from 67-84% [28]. But current study could classify only 22.5% of tumours by morphology confidently which were further confirmed by IHC. For the remaining 77.4% showed ambiguous morphology which were grouped as NSCLC with ambiguous morphology which was further subtyped with help IHC reducing NSCLC-NOS category significantly with a p-value <0.001. Overall findings of the study are in keeping with previously refined diagnostic conclusions drawn by Pelosi G et al., and Walia R et al., both of which could accurately classify 89% and 85.5% of NSCLC into ADC or SCC, respectively based on two hit, sparing material approach [12,13]. Present study classified 86.1% of NSCLC with ambiguous morphology into ADC or SCC. Many previous studies evaluated the utility of three or more markers and found a similar diagnostic rate as present study which involved two marker minimalistic panel [29-31].

LIMITATION(S)
Lack of confirmatory methods like resection specimens, complete immunopanel or genetic profiling for further categorisation of NSCLC-NOS category.

CONCLUSION(S)
There is significant improvement in subtyping of NSCLC into ADC and SCC by using TTF1/p40 minimalistic immunopanel which shows the diagnostic assessment carried out by IHC would have better relevance, reliability and weightage to the patient clinical management compared to morphological diagnosis alone. This study provides evidence on diagnostic performance of two-hit minimalistic IHC approach on small biopsy samples based on TTF1/p40 panel that is not only diagnostically helpful but also material sparing for molecular testing.

REFERENCES