p40 & thyroid transcription factor-1 immunohistochemistry: A useful panel to characterize non-small cell lung carcinoma-not otherwise specified (NSCLC-NOS) category

Ritika Walia1, Deepali Jain1, Karan Madan2, Mehar C. Sharma1, Sandeep R. Mathur1, Anant Mohan2, Venkateswaran K. Iyer1 & Lalit Kumar3

Departments of 1Pathology, 2Pulmonary Medicine & Sleep Disorder & 3Medical Oncology, All India Institute of Medical Sciences, New Delhi, India

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Background & objectives: Accurate histopathological subtyping of non-small cell lung carcinoma (NSCLC) is essential for targeted therapeutic agents. Immunohistochemistry (IHC) is helpful in identification of different tumour subtypes. In this study two marker approaches, one each for glandular and squamous cell differentiation was applied to maximize the proportion of accurately subtyped NSCLC not otherwise specified (NOS) tumours on small biopsy samples.

Methods: Two hundred and sixty three consecutive lung biopsies of primary lung carcinoma were prospectively studied. These were subtyped first morphologically and then by IHC for p40 and thyroid transcription factor-1 (TTF-1). The diagnosis of NSCLC-NOS before and after addition of IHC was evaluated. Results were correlated and validated with morphologically proven cases and matched surgical specimens.

Results: Based on morphology, only 140 of the 263 (53.2%) cases of NSCLC were characterized, whereas 123 (46.7%) were classified as NSCLC-NOS type. With addition of IHC (p40 and TTF-1), the latter category reduced to 14.4 per cent and a sum of 225 (85.5%) cases were accurately subtyped into squamous cell carcinoma, adenocarcinoma and adenosquamous carcinoma. p40 showed 100 per cent sensitivity and specificity for squamous differentiation whereas TTF-1 showed sensitivity of 85.3 per cent and specificity of 98.1 per cent. Ninety per cent correlation of morphologic subtypes was achieved with matched resected specimens.

Interpretation & conclusions: Our results showed that an approach of using only a two-antibody panel (p40 and TTF-1) might help in reduction of diagnostic category of NSCLC-NOS significantly and contribute in saving tissue for future molecular testing.

Key words Immunohistochemistry - non-small cell lung cancer/carcinoma - NSCLC-not otherwise specified - p40 - small biopsy - thyroid transcription factor-1
Non-small cell lung carcinomas (NSCLC) account for 80 per cent of all lung cancers. The developments in the chemotherapy and targeted therapy against specific molecular alterations have necessitated precise subclassification of NSCLC which comprise adenocarcinomas (ADC) and squamous cell carcinomas (SQC) predominantly. Most patients of lung cancer are diagnosed at a late stage precluding surgical resection. Hence, small biopsies or cytology remains the mainstay for accurate diagnosis. Moreover, preserving tissue for molecular studies is important because of its therapeutic implications. Earlier studies have described the usefulness of immunohistochemistry (IHC) panels consisting of various markers such as thyroid transcription factor (TTF-1), napsin-A and cytokeratin 7 (CK7) for ADC and p63, CK5/6, CK34bE12, desmoglein-3 or desmocollin for SQC for subtyping of NSCLC. Others have recommended the use of a limited panel of immunohistochemical markers comprising one squamous marker such as p40 and one marker of glandular differentiation such as TTF-1. p40 has been shown to be superior to the commonly used antibody p63 (clone 4A4) for squamous differentiation as p63 can be found in some lung ADCs and even large cell lymphomas, making it less specific. The present study was undertaken to evaluate the utility of two marker approach one each for squamous cell (p40) and ADC (TTF-1) for accurate characterization of NSCLCs. In addition, for validation purposes of these two immunostains on small biopsies, IHC was also performed in morphologically identifiable SQC and ADC and results were compared.

### Material & Methods

This prospective study was conducted in the department of Pathology, All India Institute of Medical Sciences, New Delhi, India, and included a series of 263 consecutive lung biopsies (January 2013 to April 2014) from patients of suspected primary lung cancer attending the departments of Pulmonary Medicine and Sleep Disorders and Medical Oncology. Of these, 145 were computed tomography-guided Tru-cut biopsies and 118 were obtained bronchoscopically. Biopsies having inadequate tumour tissue for immunohistochemical evaluation were excluded. None of these patients had any clinicoradiological evidence of primary cancer elsewhere. All biopsies were stained by standard haematoxylin and eosin stain and extra sections were cut on adhesive coated slides for IHC to prevent loss of tissue due to repeated facing of the block. Corresponding resection specimens were available for correlation of morphology and immunohistochemical results in 20 patients. The tumours were initially evaluated by morphological examination. The International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) classification criteria were used to determine histological subtypes of NSCLC. Small cell carcinomas, other neuroendocrine tumours and large cell neuroendocrine carcinomas were excluded on the basis of classical morphological features.

The study protocol was approved by the institutional ethics committee and written informed consent was obtained from all patients.

### Morphological examination: All NSCLC were diagnosed as SQC when characteristic intercellular bridges and keratin pearls were present. Adenocarcinomas were diagnosed when there was a definite evidence of glandular differentiation and/or intracellular mucin vacuoles. Diagnosis of adenosquamous carcinoma was made when foci of both squamous and glandular differentiation were identified. All other cases showing solid growth pattern or lacking any definite differentiation were classified as NSCLC-not otherwise specified (NOS) by morphology. Cases with morphological features such as organoid pattern of growth suggestive of large cell neuroendocrine carcinomas were excluded.

### Immunohistochemistry (IHC): IHC for p40 and TTF-1 was performed in all resected specimens and biopsies. The latter consisted of two groups including morphologically identifiable NSCLC and NSCLC-NOS. The cases were designated as NSCLC favour ADC if the immune profile was p40−/TTF1+, NSCLC favour SQC if p40+/TTF1− and NSCLC favour adenosquamous carcinoma if p40+/TTF1+ in different tumour cells. TTF-1 (mouse anti-human TTF-1 monoclonal antibody, clone 8G7G3/1 from Spring Bioscience Pleasanton, California) in 1:50 dilution and p40 (PC373 anti-p40 rabbit polyclonal antibody, from Calbiochem, Darmstadt, Germany) in 1:3000 dilutions were used. Briefly, after blocking endogenous peroxidase activity in 0.3 per cent hydrogen peroxidase and methanol solution for 15 min, deparaffinized sections were reacted for 30 min at room temperature with primary antibody.Slides were then incubated in...
a detection kit (EnVision plus HRP, DakoCytomation, Glostrup, Denmark) according to manufacturer’s instructions, developing peroxidase activity with 3-3'-diaminobenzidine. Antigen retrieval was performed using citric acid (pH 6.0). Finally, slides were counterstained with haematoxylin, dehydrated and mounted.

Immunoreactivity was rendered semi-quantitatively on a scale from 0 to 3+, which was calculated as follows: percentage positivity of cells was graded as 0, 1 (1-25%), 2 (25-50%) and 3 (50-100%) and intensity graded as 0 (no staining), 1 (weak), 2 (moderate) and 3 (strong). The score was added and calculated on a scale of 0-9 (0-1=0, 2-4=1+, 5-7=2+, 8-9=3+). Care was taken not to interpret entrapped normal bronchial epithelium or pneumocytes as positive for tumour cell staining. Morphological assessment and IHC interpretation were performed independently by two pathologists at different time.

Statistical analysis: McNemar’s test was performed to assess the significance of the correlation in the diagnosis of NSCLC-NOS before and after addition of IHC. The specificity and sensitivity of IHC markers was calculated and reported. GraphPad Prism software (https://www.graphpad.com/scientific-software/prism/) was used to quantify agreement with kappa.

Results

The median age of the patients was 58 yr with an interquartile range of 50-65 years. Male to female ratio was 3:1. Seventy per cent of the tumours were ADC in females whereas ADC constituted 45.9 per cent of all tumours in males. Males had a higher percentage of SQC and NSCLC-NOS as compared to females. All adenosquamous carcinomas were found in males.

Based on the morphological analysis, 54 (20.5%) cases could be classified as SQC, 82 (31.1%) as ADC and four (1.5%) as adenosquamous carcinoma. The remaining 123 (46.7%) cases could not be further subtyped and were labelled as NSCLC-NOS.

Morphological analysis

Squamous cell carcinoma (SQC): Of the 54 morphologically identified SQC, 25 were well differentiated and 29 were moderately differentiated. One case revealed pseudo-glandular areas due to acantholysis. Seven cases had corresponding resection specimens, all of which turned out to be morphologically differentiated SQC.

Adenocarcinomas (ADC): Eighty two morphologically identified ADCs showed focal glandular differentiation and intracytoplasmic mucin. Five of these had corresponding resection and turned out to be well to moderately differentiated ADC.

Adenosquamous carcinoma: Four of the 263 cases had definite squamous and glandular areas on morphology and were diagnosed as adenosquamous carcinoma. None of these had corresponding resection specimen.

Immunohistochemical analysis

p40: All morphologically identified SQC were p40 positive (Fig. 1A and B). All cases showed 2+ to 3+ positivity except for two cases, which showed 1+ positivity. The staining intensity was strong in all cases. One case showing pseudoglandular architecture revealed 3+ positivity of p40 with no staining for TTF-1 (Fig. 1C and D).

Thyroid transcription factor-1 (TTF-1): Of the 82 morphologically diagnosed ADC, TTF-1 was positive (2+ to 3+) in 70 cases (85.3%) (Fig. 2A and B) whereas negative in 12 (14.6%) cases. TTF-1 was positive (3+) in one (1.8%) SQC along with p40 in the same tumour cells (sensitivity 85.3% and specificity 98.1%).

Adenosquamous carcinoma and immunohistochemistry: Amongst adenosquamous carcinomas, all four cases exhibited focal p40 and TTF-1 expression in different tumour cell populations (Fig. 2C-E).
NSCLC-NOS: One hundred and twenty three cases did not show any morphological differentiation (Fig 3A-D) (Table I). These were classified on the basis of IHC. Detailed immunophenotypic characterization is shown in Table II. IHC was negative for both the markers in 38 (14.4%) cases, which remained NSCLC-NOS and could not be subtyped further.

As a result, IHC for two specific markers when used adjunctively with morphology was able to correctly classify 225 of the 263 (85.5%) cases of NSCLC ($P<0.001$) (Table III).

Resection specimens: Corresponding resection specimens were available for 20 cases. Original diagnosis and immunohistochemical profile on small

![Fig. 2.](image)

**Table I.** Utility of adjunctive immunohistochemistry in subtype characterization of non-small cell lung cancer (NSCLC)

| Tumour type              | Morphological diagnosis (n=263) n (%) | After using IHC (p40 and TTF-1) n (%) | Subtype characterization |
|--------------------------|--------------------------------------|--------------------------------------|--------------------------|
| ADC                      | 82 (31.1)                            | 137 (52)                             | Increment of 20.9%       |
| SQC                      | 54 (20.5)                            | 80 (30.4)                             | Increment of 9.9%        |
| Adenosquamous carcinoma  | 4 (1.5)                              | 8 (3)                                | Increment of 1.5%        |
| NSCLC-NOS                | 123 (46.7)                           | 38 (14.4)                             | Reduction of 32.3%       |

IHC, immunohistochemistry; TTF-1, thyroid transcription factor 1; ADC, adenocarcinomas; SQC, squamous cell carcinomas; NOS, not otherwise specified

**Table II.** Results of immunohistochemistry combination of p40 and thyroid transcription factor-1 (TTF1)

| IHC                  | Morphology                | ADC (n=82) | SQC (n=54) | Adenosquamous carcinoma (n=4) | NSCLC-NOS (n=123) |
|----------------------|---------------------------|------------|------------|-----------------------------|-------------------|
| P40+/TTF-1-          |                           | 0          | 53         | 0                           | 26*               |
| P40-/TTF-1+          |                           | 70         | 0          | 0                           | 55*               |
| P40-/TTF-1-          |                           | 12         | 0          | 0                           | 38*               |
| P40+/TTF-1+          |                           | 0          | 1          | 4                           | 4*                |

*NSCLC favour SQC; +NSCLC favour ADC; +NSCLC-NOS; ^NSCLC favour adenosquamous carcinoma. IHC, immunohistochemistry; NSCLC, non-small cell lung cancer; ADC, adenocarcinomas; SQC, squamous cell carcinomas; NOS, not otherwise specified
biopsies correlated well with final diagnosis in 18 of the 20 cases. One case classified as ADC on biopsy turned out to be adenosquamous on the surgical specimen while other cases classified as NSCLC-NOS was sarcomatoid carcinoma with CK, vimentin and focal TTF-1 positivity (Table IV).

Of the seven ADCs on resection specimens; four were acinar predominant and three were lepidic predominant. SQCs were mostly keratinizing (6/11; 54.5%) type. No basaloid variant was recognized. Sarcomatoid carcinoma was of pleomorphic subtype due to focal glandular differentiation (TTF-1 positivity) and predominantly spindle cell areas. On biopsy, this case did not show spindle cells whereas TTF-1 and p40 negative solid tumour cell fragments were observed, thus labelled as NSCLC-NOS.

**Discussion**

This study was conducted with the aim of studying the effectiveness of a minimal panel of antibodies comprising p40 and TTF-1 in subclassification of NSCLC. Morphology alone is not very sensitive to precisely classify NSCLC into squamous or ADC in small biopsies. The error rate in classifying NSCLC into appropriate histological type is high in small biopsies because of sampling of solid areas of the tumour lacking morphologic differentiation. In one such study, 10 per cent of SQC, 14 per cent of ADC and 50 per cent of large cell carcinomas were misclassified on bronchial biopsies. The frequency of correct diagnosis of NSCLCs on small biopsies by light microscopy varies from 67 to 84 per cent. We could classify only 53 per cent of tumours by morphology confidently which were further confirmed by IHC. The remaining 46.7 per cent were classified as NSCLC-NOS by morphology and further subtyped with the aid of IHC reducing the diagnosis of NSCLC-NOS significantly.

Using a panel of TTF-1, p63, CK5/6 and PAS (periodic acid-Schiff)-diastase, NSCLCs could be characterized in 65 per cent of cases. A cocktail of nuclear and cytoplasmic stains, such as TTF-1/napsin-A and p63/CK5/6 was also suggested as a useful marker combination. A limitation of availability of adequate

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**Table III.** Significance of adding immunohistochemistry in the diagnosis of non-small cell lung cancer (NSCLC) (n=263)

| Tumor type                                          | Morphology (%) | Addition of IHC (%) | P     |
|-----------------------------------------------------|----------------|---------------------|-------|
| NSCLC-NOS                                           | 123 (46.7)     | 38 (14.4)           | <0.001|
| Subtypes (ADC, SQC and adenosquamous carcinoma)      | 140 (53.2)     | 225 (85.5)          |       |

NOS, not otherwise specified; ADC, adenocarcinomas; SQC, squamous cell carcinomas; IHC, immunohistochemistry

**Table IV.** Correlation of immunohistochemical diagnosis on biopsy with final diagnosis on surgical specimen

| Diagnosis on resection specimen | Number of cases (n=20) | Original diagnosis on biopsy | ADC | NSCLC favour ADC | SQC | NSCLC favour SQC | NSCLC-NOS |
|--------------------------------|------------------------|-------------------------------|-----|------------------|-----|------------------|-----------|
| ADC                            | 7                      |                               | 4   | 3                | 0   | 0                | 0         |
| SQC                            | 11                     |                               | 0   | 0                | 7   | 4                | 0         |
| Adenosquamous carcinoma         | 1                      |                               | 1   | 0                | 0   | 0                | 0         |
| Sarcomatoid carcinoma           | 1                      |                               | 0   | 0                | 0   | 0                | 1         |

NSCLC, non-small cell lung cancer; ADC, adenocarcinomas; SQC, squamous cell carcinomas; NOS, not otherwise specified
material in a small biopsy precludes the usage of an extensive panel of immunohistochemical markers to accurately subtype NSCLCs, especially when tissue needs to be saved for molecular studies. Hence, it has been proposed to use one specific marker each for squamous and glandular differentiation\textsuperscript{9,17-20}. p40 has recently entered the diagnostic milieu of NSCLCs with only a few studies evaluating its role. All these studies considered p40 a single best marker for identifying SQC\textsuperscript{9,17-20}. Many studies have utilized three or more markers for subclassification and have found a similar diagnostic rate as the present study where only two markers have been used\textsuperscript{20-22}. There are only a few studies exploring the ‘two marker’ minimalistic approach through coordinated use of single marker including p40 and TTF-1 for NSCLC subtyping in small biopsies\textsuperscript{9}.

p40 is equivalent to p63 in terms of sensitivity but is superior in terms of specificity as it is negative in p63 positive ADC\textsuperscript{11,23}. p40 was positive in all morphologically differentiated SQC in our study and negative in all ADC. p40 also decorates the squamous component of adenosquamous carcinoma\textsuperscript{24}. There were eight cases of adenosquamous carcinoma in our study all of which were focally positive for p40 defining the squamous component. Four of these cases were diagnosed in retrospect after IHC revealed focal p40 positivity.

TTF-1 is an established marker for lung ADC. However, its recorded sensitivity varies from 70 to 96 per cent\textsuperscript{8,25}. TTF-1 was observed as a sensitive and specific marker (sensitivity 85.3\% and specificity 98.1\%) for glandular differentiation.

The coexistence of glandular and squamous traits within the same tumour cells are described in pulmonary\textsuperscript{26} and oesophageal\textsuperscript{27} carcinomas as a possibility of ‘amphicrine’ bi-phenotypic tumours. We found one morphologically differentiated SQC, in which p40 and TTF-1 were positive in the same tumour cells.

Negativity of TTF-1 in the absence of p40 reliably excludes SQC and may imply a poorly differentiated ADC. An unsuspected metastatic carcinoma, however, needs to be excluded in such cases by careful clinical investigation. Pan-CK stain should be performed to confirm the epithelial origin if morphologically tumour appears non-epithelioid.

Coordinated application of p40/TTF-1 immunopanel has been found to be useful in our study to significantly lower down the rate of NSCLC-NOS in small biopsies, and this panel was diagnostic in 85.5 per cent of cases. Amongst NSCLC-NOS category itself, this panel was helpful in diagnosing 70 per cent of tumours. The diagnosis on resected specimens correlated well with the diagnosis on biopsies in 90 per cent cases. One adenosquamous carcinoma and one sarcomatoid carcinoma escaped recognition probably because of sampling error.

In this study, CK7 was used as an additional stain for the 38 cases, which remained NSCLC-NOS after application of p40/TTF-1. Ten cases showed positivity for CK7. This was not included in the study as it was intended to evaluate the role of two-marker approach. The only limitation of the study was unavailability of matched resection specimens in all the cases. However, due to advanced stage of the disease at the time of presentation in lung cancer, resection was not found in most of the cases.

In conclusion, NSCLC characterization was performed using a combination of morphology and IHC. The two-antibody panel approach may contribute to accurate subclassing of NSCLC in small biopsies and a reduction in the NSCLC-NOS diagnostic category.

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**Conflicts of Interest:** None.

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Reprint requests: Dr Deepali Jain, Associate Professor, Department of Pathology, All India Institute of Medical Sciences, New Delhi 110 029, India e-mail: deepalijain76@gmail.com