Role of Immunohistochemistry in The Subtyping of Non Small Cell Lung Carcinoma on True Cut Lung Biopsies

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ABSTRACT

Background: Worldwide, lung carcinoma is the most common cancer in terms of number of cases and deaths. Lung carcinomas are broadly divided into small cell carcinoma and non-small cell lung carcinoma (NSCLC). In recent years availability of targeted therapies necessitates subtyping the NSCLC to improve the survival and quality of life. NSCLC can be subtyped by routine Haematoxylin and Eosin (H&E) stained section slides alone, poorly differentiated tumors are difficult to segregate morphologically, especially in true-cut biopsies, necessitating ancillary techniques like immunohistochemistry (IHC). Hence this study was taken up to examine accuracy of diagnosis of Non-Small Cell Lung Carcinoma (NSCLC) on biopsy samples initially made based on morphology and then with IHC using relevant markers like CK5, CK6, CK7, Napsin-A, TTF-1, P63, Synaptophysin and Chromogranin-A.

Methods: A prospective study of two years and six months duration during which 111 cases of NSCLC on true-cut biopsies were first reported on Haematoxylin and Eosin sections and later subjected for IHC.

Results: Out of 111 cases, after IHC, 80 were diagnosed as adenocarcinoma and 31 as squamous cell carcinoma NSCLC were common in the 6th decade. In adenocarcinoma positivity for CK7, TTF1 and Napsin-A was 95%, 75% and 78.75% respectively. In squamous cell carcinoma positivity for CK5, CK6 and P63 was 84%, 81% and 90% respectively.

Conclusion: CK5, CK6 and P63 can be used for confirming squamous cell carcinoma and CK7, TTF1 and Napsin-A for adenocarcinoma. We recommend an IHC panel consisting of P63, TTF1 and Napsin-A in NSCLC-unclassifiable and poorly differentiated carcinoma on H&E.

Keywords: Adenocarcinoma, Squamous Cell Carcinoma, CK7, CK5, CK6, P63, Napsin-A, TTF1
were processed, embedded, cut into 4µ thick sections, stained with H&E, mounted and histopathologically reported.

A total of 162 biopsies were received out of which 51 cases were excluded from the study (11 non-neoplastic cases, 8 metastatic, 5 sarcomas, 1 lymphoma, 16 small cell carcinomas and 10 inadequate biopsies) (Table-1). The remaining 111 samples were subjected to immunohistochemical staining with CK5, CK6, CK7, P63, TTF-1, Napsin-A, Synaptophysin, Chromogranin-A. The sections were deparaffinized in xylene and rehydrated through absolute alcohol.

Antigen retrieval in citrate buffer (pH 9 Lab vision cat#AP9003) was used after the sections were treated in a microwave at 8 W for 5–6 min, then at 3 W for 10 min, and the sections were then left to cool for 20 minutes. Peroxidase and protein blockades were done and the sections were later incubated overnight with the primary antibodies at room temperature using Cytokeratin5(CK5) pre-diluted rabbit monoclonal antibody (EPR24/Rabbit), Cytokeratin 6(CK6) pre-diluted rabbit monoclonal antibody (EP67/Rabbit), Cytokeratin7(CK7) pre-diluted rabbit monoclonal antibody(EPR16/Rabbit), P63 pre-diluted mouse monoclonal antibody (4A4/Mouse), TTF-1 pre-diluted mouse monoclonal antibody(8G7G3/1/Mouse), Napsin-A pre-diluted mouse monoclonal antibody(IP64/Mouse), Synaptophysin pre-diluted polyclonal antibody(rabbit), Chromogranin-A pre-diluted mouse monoclonal antibody (LK2H10/Mouse) from Biogenix company followed by rinsing in PBS (phosphate buffered saline, pH 7.6). This was followed by incubation with the secondary biotin conjugated antibody for one hour and finally incubation with peroxidase conjugated streptavidin for another hour. Diaminobenzidine tetrachloride (DAB) was added and kept for 25 minutes, and then counterstained in Haematoxylin, followed by dehydration, clearing and mounting. Positive control sections were prepared from squamous cell carcinoma of nasopharynx for CK5, CK6 and P63, papillary carcinoma of thyroid for TTF1, lung adenocarcinoma for Napsin-A, paraganglioma for synaptophysin and chromogranin-A. Negative controls were done by excluding primary antibody and its replacement with PBS. Presence (≥1% positivity in lesional cells) and absence of immunoreactivity in tumor cells were given 1 & 0 points respectively. 

Statistical Analysis: Each case data was entered in a Microsoft Excel 2007, spread sheet (Microsoft Corp, Redmond, WA). Fisher’s exact test (SPSS20 VERSION) was used for analysis of contingency tables with significance level at p<0.05.

Results
The subjects were aged between 24 years to 85 years. The youngest was a 24-year-old male and oldest was an 85-year-old female. Majority of the patients were in 6th decade (41 cases, 37%) followed by 7th (36 cases, 32.5%), 8th (19 cases, 17%), 5th (10 cases, 9%) and 9th decade (3 cases, 2.7%) and one each (0.9%) in the 3rd and 4th decades. The male: female ratio is 3.5:1. The mean age group is 61.66 ± 10.03 years.

Out of 111 cases, adenocarcinomas were 80 (72%) with maximum cases being in the 6th decade and squamous cell carcinomas were 31 (28%) with maximum cases being in the 7th decade (12), with male preponderance seen in both adenocarcinoma (M: F-3:1) and squamous cell carcinoma (M: F-7:1). Initial diagnosis on H&E of adenocarcinoma, 62 cases, and squamous cell carcinoma, 14 cases, were confirmed after IHC. Out of 21 cases initially diagnosed as NSCLC (unclassifiable) on H&E, 12 cases were diagnosed as adenocarcinoma and 9 cases were diagnosed as squamous cell carcinoma after IHC. Out of 14 cases which were initially diagnosed as poorly differentiated carcinoma on H&E, 6 cases were diagnosed as adenocarcinoma and 8 cases were diagnosed as squamous cell carcinoma after IHC (Table 2).

The individual IHC marker positivity among the adenocarcinoma cases (n=80) is given in Table 3. Among the 80 cases, 60 cases were positive for CK7, Napsin-A, and TTF-1, 11 cases were positive for CK7 and Napsin-A in, 5 cases were positive for CK7 and TTF-1 and 2 cases were positive for TTF-1 and Napsin-A.

The individual IHC marker positivity among the squamous cell carcinoma cases (n=31) is given in Table 4. Among the 31 cases, 22 cases were positive for CK5, CK6, P63, 2 cases were positive for CK6 and P63, 1 case each for CK5, P63 and CK5, CK6. P63 alone was positive in 3 cases and 2 cases were positive only for CK5.

Discussion
In India, lung cancer constitutes 6.9 per cent of all new cancer cases and 9.3 per cent of all cancer related deaths in both sexes. It is the most common cause of cancer related mortality in men. Surgery is the treatment of choice for early stage of lung cancer and radiotherapy with or without chemotherapy is the treatment for inoperable stage I, stage II and stage III cases. The overall 5-year survival rate of lung cancer is dismal with approximately 15 per cent in developed countries and 5 per cent in developing countries. In the Western countries and most of the Asian countries adenocarcinoma presentation has surpassed the previous common observations of squamous cell carcinoma.
Table 1: Total lung biopsy specimens received.

| LESIONS                                | NUMBER OF CASES (n) |
|----------------------------------------|---------------------|
| INCLUDED CASES                         |                     |
| Non-small cell lung carcinomas         | 111                 |
| Non-neoplastic lesions                 | 11                  |
| Metastatic lesions                     | 8                   |
| Sarcomas                               | 5                   |
| Lymphomas                              | 1                   |
| Small cell carcinomas                  | 16                  |
| Inadequate                             | 10                  |
| TOTAL                                  | 162                 |

Table 2: The initial diagnosis made on H&E stained slides and final diagnosis made after IHC.

| DIAGNOSIS                                      | NUMBER OF CASES (n) | ADENOCARCINOMA | SQUAMOUS CELL CARCINOMA |
|------------------------------------------------|---------------------|----------------|-------------------------|
| Adenocarcinoma                                 | 62                  | 62             | 0                       |
| Squamous cell carcinoma                        | 14                  | 0              | 14                      |
| Non-small cell lung carcinoma unclassifiable   | 21                  | 12             | 9                       |
| Poorly differentiated carcinoma                 | 14                  | 6              | 8                       |
| **Total**                                      | **111**             | **80**         | **31**                  |

Table 3: Positivity and negativity including percentage of IHC markers in adenocarcinoma.

|         | CK5    | CK6    | P63    | CK7    | TTF1   | NAPSIN-A | SYN    | CHROM A |
|---------|--------|--------|--------|--------|--------|----------|--------|---------|
| **POSITIVE n(%)** | 12(15%) | 15(18.75%) | 7(8.75%) | 76(95%) | 60(75%) | 63(78.75%) | 5(6.25)% | 0(0%) |
| **NEGATIVE n(%)**  | 68(85%) | 65(81.25%) | 73(91.25%) | 4(5%) | 20(25%) | 17(21.25%) | 75(93.75%) | 31(100%) |

Table 4: Positivity and negativity including percentage of IHC markers in squamous cell carcinoma.

|         | CK5    | CK6    | P63    | CK7    | TTF1   | NAPSIN-A | SYN    | CHROM-A |
|---------|--------|--------|--------|--------|--------|----------|--------|---------|
| **POSITIVE n(%)** | 26(84%) | 25(81%) | 28(90%) | 9(35.5%) | 2(6.5%) | 0(0%) | 2(6.5%) | 0(0%) |
| **NEGATIVE n(%)**  | 5(16%) | 6(19%) | 3(10%) | 20(65%) | 29(93.5%) | 31(100%) | 29(93.5%) | 31(100%) |
Fig. 1A: Photomicrograph showing adenocarcinoma with cuboidal cells with mild nuclear pleomorphism arranged in the form of glandular exhibitions-true-cut lung biopsy(H&E20X) Fig1B: Photomicrograph showing diffuse cytoplasmic positivity of cytokeratin7 in the lesional cells of true-cut lung biopsy diagnosed as adenocarcinoma after ihc.(IHC-20X). Fig 1C: Photomicrograph showing diffuse nuclear positivity of TTF-1 in the lesional cells of true-cut lung biopsy diagnosed as adenocarcinoma after ihc.(IHC-20X). Fig1D: Photomicrograph showing diffuse granular cytoplasmic positivity of Napsin-A in the lesional cells of true-cut lung biopsy diagnosed as adenocarcinoma after ihc.(IHC-20X).

Fig. 2A: Photomicrograph showing focal cytoplasmic CK5 positivity in adenocarcinoma-true-cut lung biopsy. (IHC-20X) Fig2B: Photomicrograph showing focal cytoplasmic CK6 positivity in adenocarcinoma-true-cut lung biopsy. (IHC-20X) Fig2C: Photomicrograph showing focal nuclear P63 positivity in adenocarcinoma-true-cut lung biopsy. (IHC-20X) Fig2D: Photomicrograph showing focal cytoplasmic Synaptophysin positivity in adenocarcinoma-true-cut lung biopsy. (IHC-20X).
Fig. 3A: Photomicrograph showing squamous cell carcinoma with islands of tumor cells with pleomorphic hyperchromatic nuclei and abundant eosinophilic cytoplasm-true-cut lung biopsy (H&E 20X).

Fig 3B: Photomicrograph showing diffuse cytoplasmic positivity of CK5 in the lesional cells of true-cut lung biopsy diagnosed as squamous cell carcinoma after ihc. (IHC- 20X).

Fig 3C: Photomicrograph showing diffuse cytoplasmic positivity CK6 in the lesional cells of true-cut lung biopsy diagnosed as squamous cell carcinoma after ihc. (IHC- 20X).

Fig 3D: Photomicrograph showing diffuse nuclear positivity of P63 in the lesional cells of true-cut lung biopsy diagnosed as squamous cell carcinoma after ihc. (IHC- 20X).

Fig. 2A: Photomicrograph showing focal cytoplasmic CK5 positivity in adenocarcinoma-true-cut lung biopsy. (IHC-20X)

Fig 2B: Photomicrograph showing focal cytoplasmic CK6 positivity in adenocarcinoma-true-cut lung biopsy. (IHC-20X)

Fig 2C: Photomicrograph showing focal nuclear P63 positivity in adenocarcinoma-true-cut lung biopsy. (IHC-20X)

Fig 2D: Photomicrograph showing focal cytoplasmic Synaptophysin positivity in adenocarcinoma-true-cut lung biopsy. (IHC-20X).
In the present study among the 111 primary lung carcinomas studied, the male: female ratio is 3.5:1, with majority of the patients in 6th decade similar to observations of Kou et al., Sheik et al., Jindal and Behera. There are studies reporting adenocarcinoma in younger age both in men and women. Histological subtypes in the final diagnosis of present study include adenocarcinoma and squamous cell carcinoma, with adenocarcinomas being the most common, which is in agreement with recent Indian studies, and in contrast to some Indian studies.

**IHC IN Adenocarcinoma:** CK7, an intermediate filament protein, weighs about 54KD, is a type2 keratin of simple non-keratinizing epithelium, found in breast, lung, ovarian, pancreatico-biliary malignancies and transitional cell carcinomas. In the present study, out of 80 cases of adenocarcinoma (Fig-1A), CK7 is positive in 76 cases (95%) (Fig-1B) similar to the findings of Gurd et al. and Sterlacci et al. (97.2%). Studies have also reported 100% positivity, 80.18% and 84% positivity. In present study, CK7 is negative in 4 cases, among them 2 cases are negative for all the 8 markers, but morphology of the tumour revealed glandular exhibitions. Hence the diagnosis of adenocarcinoma which was made on morphology could not be excluded in these two cases.

TTF-1 is a nuclear transcription factor that is expressed in epithelial cells of lung and thyroid. In the lung, it regulates the expression of genes involved in the production of surfactant. TTF-1 is also immunoreactive in other tumors like thyroid neoplasms, breast adenocarcinoma, gastro intestinal carcinoma, small cell carcinoma, carcinoid and possibly controversially in squamous cell carcinoma of lung. Kargi et al. reported that in small cell carcinoma sometimes it will be positive for TTF-1 and negative for P63. In present study, TTF-1 is positive in 60 adenocarcinoma cases (75%) (Fig-1C) which is higher when compared to the studies done by Kargi et al. (40%), Sainz et al. (63%) and in agreement with Modi et al. (74%), Bishop JA et al. (73%).

Higher percentage of positivity has been reported by Mukopadhyay S et al. (80%) (80%), Zhao et al. (80%) and Gurd et al. (84.5%) and Sterlacci et al. (83.5%) and Yaman et al. (85%), Burnstrom et al. (92%) and Xuxy et al. (94.5%). The reason for above variability in positivity for TTF-1 could be because TTF-1 will be more positive in better differentiated adenocarcinoma. Of 20 cases of adenocarcinomas which were TTF-1 negative, 17 cases were CK7 positive, 7 cases were positive for Napsin-A, 3 cases were CK7 negative and 11 cases were negative for Napsin-A.
variable immunostaining for CK6. Out of 15 cases showing positivity for CK6, 9 cases showed positivity for CK7, TTF-1 and Napsin-A. Positivity for CK7 alone was seen in 2 cases. 3 cases were positive for CK7 & Napsin-A. 3 cases were negative for TTF-1 alone and 2 cases were negative for both TTF-1 and Napsin-A.

Human TP63 gene is located on the chromosome 3q27-29 and the expression of the gene produces the full-length protein P63 and the truncated P40. P63 can be detected in benign bronchial stem cells and in neoplastic cells with evidence of squamous differentiation. P63 will be positive in metastatic urothelial carcinoma, myoepithelial neoplasms, lymphoma and sarcoma. P63 can be expressed in 21.7% of adenocarcinoma. A 10-65% positivity of P63 in adenocarcinoma was reported where it shows weak and focal expression in contrast to strong and diffuse expression in squamous cell carcinoma. It has been shown that P63 expression will be seen in poorly differentiated adenocarcinoma which are negative for TTF-1 and Napsin-A. Several studies showed that P63 is consistently positive in lung squamous cell carcinoma irrespective of the degree of differentiation. In present study, P63 is positive in 7(8.75%) cases(Fig-2C), similar to Sterlacci et al (7%) and Mukopadhyay S et al (10%).

Studies done by Burtnstrom et al [20](26%) and Zhao et al [21](16%) revealed high percentage of positivity. Out of 7 cases of adenocarcinomas showing P63 positivity, 6 cases showed positivity for CK7, TTF-1 and Napsin-A and in one case patchy positivity for P63 was seen in association with diffuse TTF-1 positivity. Yaman et al [27], Modi et al [14] and Kargi et al [23] did not find P63 positivity.

Synaptophysin in the present study is positive in 5(6.25%) cases(Fig-2D) which is nearly equal to the study done by Burtnstrom et al [20] (7%). Out of 5 cases of adenocarcinoma showing positivity for synaptophysin, 4 cases were positive for CK7, TTF-1, Napsin-A except for 1 case which is negative for TTF-1 and positive for CK7 and Napsin-A.

Chromogranin-A was negative in all of the cases (0%) in the present study.

**Squamous Cell Carcinoma (Fig-3A)** CK5 was found to be positive in 26(84%) cases(Fig-3B) which is slightly higher than Zhao et al [21] (81.25%). Mukopadhyay S et al [26] reported positivity of 73%, Xuay et al [22], Burtnstrom et al [20] and Sterlacci et al [19], revealed higher percentage of positivity 91.92%, 98% & 99.2% respectively. Gurda et al [18] reported 100% positivity. Out of 31 cases of squamous cell carcinomas, 26 cases showed positivity for CK5 and 5 cases were negative. All the CK5 negative cases are positive for P63 and only 2 CK5 negative cases were positive for CK6.

CK6 is positive in 25(81%) cases(Fig-3C), similar to Zhao et al [21] (81.25%). In the 6 cases negative for CK6, 2 cases were positive for CK5 alone, 2 cases for P63 alone, 1 case for both CK5 and P63 and 1 case for P63 and CK7. Studies by Sterlacci et al [19] (99.2%), Xuay et al [22] (91.92%) and Mukopadhyay S et al [26] (73%) reported varying percentages of positivity. Gurda et al [18] reported 100% positivity.

P63 is positive in 28(90%) cases(Fig-3D) in our study similar to Yaman et al [27] (87.5%), Gurda et al [18] (91.75%), Xuay et al [22] (96.95%), Burtnstrom et al [20] (97%) and Sterlacci et al (99.2%). Mukopadhyay S et al [26] and Zhao et al [21] reported 100% positivity. Kargi A et al [23] and Modi et al [14] revealed 61% and 82.05% positivity. Among the 3 P63 negative cases one was positive for CK5 and CK6 and 2 were positive for only CK5.

CK-7 is positive in 11 (35.5%) cases in the present study (Fig-4A). Out of 11 cases showing positivity for CK7, 8 showed positivity for CK5, CK6 & P63, 1 case showed positivity for CK5 and CK6 and 2 cases showed positivity for only P63. Varying percentages are reported by Sterlacci et al [19] (23.5%), Burtnstrom et al [20] (46%), Gurda et al [18] (50%), Modi et al [14] (57%) and Zhao et al [21] (62.5%).

TTF-1 is positive in 2(6.5%) cases(Fig-4B) which is higher than Sterlacci et al [19] (3.4%). In these 2 cases of squamous cell carcinoma, CK5, CK6 and P63 showed positivity. Modi et al [14], Kargi et al [23], Mukopadhyay S et al [26], Burtnstrom et al [20] and Zhao et al [21] did not find positivity for TTF-1.

Napsin-A is negative in all the cases (0%) similar to Zhao et al [21] (0%). Burtnstrom et al [20] found 5% positivity.

Synaptophysin is positive in 2(6.5%) cases, higher than Burtnstrom et al [20] (2%). In these 2 cases CK5, CK6 and P63 were positive. In 1 case in addition to these 3 markers, CK7 positivity is also seen.

Chromogranin-A is negative in all the cases (0%) in the present study. Although not common, positive neuroendocrine markers do not rule out the diagnosis because few cases of adenocarcinomas, squamous cell carcinomas and non-neuroendocrine large cell carcinomas showed positivity for neuroendocrine markers in other studies.

In 12 cases of NSCLC- unclassifiable which turned out to be adenocarcinomas after IHC, 5 cases showed positivity for CK7, TTF-1 and Napsin-A, 2 cases positive for CK7, TTF-1, 2 cases positive for CK7 and Napsin-A and 3 cases are positive for TTF-1 and Napsin-A. In 6 cases of poorly differentiated carcinomas which turned out to be
adenocarcinomas after IHC, 5 cases showed positivity for CK7, TTF1 and Napsin-A and 1 case showed positivity for CK7 and TTF1. In 9 cases of NSCLC-unclassifiable which turned out to be squamous cell carcinomas after IHC, 5 cases showed positivity for CK5, CK6 and P63, 1 case positive for CK5 and P63, 1 case positive only for P63 and 2 cases positive for CK5 and CK6. In 8 cases of poorly differentiated carcinomas which turned out to be squamous cell carcinomas after IHC, 6 cases showed CK5and P63 positivity, 2 cases showed only P63 positivity.

CK5, CK6 and P63 (p<0.001) may help in detecting squamous cell carcinoma and CK7, TTF-1, Napsin-A(p<0.001) may help in detecting adenocarcinoma. Synaptophysin (p=1.00) is not helpful in detecting either adeno and squamous cell carcinoma.

Conclusion
A panel of IHC markers are helpful to differentiate adenocarcinoma and squamous cell carcinoma on small true-cut lung biopsies which can influence the personalized treatment of the patient. CK5, CK6 and P63 help in detecting squamous cell carcinoma, CK7, TTF-1 and Napsin-A for detecting adenocarcinoma. In NSCLC-unclassifiable and poorly differentiated carcinoma as diagnosis on H&E, we recommend a IHC panel consisting of P63, TTF-1 and Napsin-A for a definitive diagnosis. From our study, we infer that Napsin-A will be negative in squamous cell carcinoma.

Limitations
Our study had few limitations like small sample size and most of the patients were in advanced stage at the time of presentation. Neither follow up of every patient for clinical improvement, resected lung specimens nor autopsy findings for final histopathology reports were available. Hence sensitivity, specificity, positive predictive value and negative predictive value could not be calculated in the present study.

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