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Cytologic subtyping of lung carcinomas on Endobronchial ultrasound guided transbronchial needle aspiration (EBUS-TBNA) samples: Role of limited panel immunocytochemistry (ICC) as an adjunct to cytomorphology

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ABSTRACT

Introduction: Majority of lung cancer presents at an advanced unresectable stage, emphasizing the diagnosis on cytological specimens like EBUS-TBNA. This study was aimed to subtype lung carcinomas on EBUS-TBNA specimens on smears and cell blocks (CB) and to assess the utility of limited panel ICC towards this subtyping.

Materials and Methods: This is both retrospective and prospective study done on total of 142 cases for two and half years. The samples from either lung or mediastinal lymph nodes diagnosed as lung carcinoma on cytomorphology in correlation with clinico – radiological features were included. Extrapulmonary metastatic tumors, lymphomas and malignancies other than carcinomas were excluded. The cytologic material (EBUS-TBNA smears and CB) was obtained by EBUS TBNA procedure done on lung lesions and/or mediastinal lymph nodes and stained with required stains. All cases were first classified as small cell carcinoma (SCC) and non small cell carcinoma (NSCC) on cytomorphology as per 2004 WHO classification. NSCC was then further subtyped. ICC with TTF1/p63 antibodies was applied on NSCC and synaptophysin on SCC/neuroendocrine tumors. Napsin A was applied on p63/TTF1 negative cases. The results were expressed in frequencies and percentages.

Result: On cytomorphology alone, 25 of 142 cases (17.6%) were classified as SCC and 117 cases (82.4%) as NSCC. NSCC was further subtyped on cytomorphology (smears+CB) into definitive (squamous cell carcinoma [SqCC]/adenocarcinoma [ADC]) v/s favouring (favouring SqCC/ ADC/ adenosquamous carcinoma) v/s not otherwise specified (NOS) in 41 (35.1%) v/s 33(28.2%) v/s 43(36.7%) cases respectively. Immunostains TTF1/ p63 and napsin A applied on possible 86 NSCC cases reduced NOS to 9.3 %. Synaptophysin confirmed all 20 cases of SCC and 1 large cell neuroendocrine carcinoma (LCNC). Overall in 142 cases, limited panel ICC reduced NSCC-NOS to 11.9%.

Conclusion: Subtyping of lung carcinoma on EBUS- TBNA samples is feasible. Preparation of cell block allows application of ICC. A limited panel ICC comprising p63, TTF1 and synaptophysin can classify in majority of cases with napsin A useful as secondary antibody in p63, TTF1 negative cases. IntroductionThe aim of this study was to know the efficacy of cytomorphology (smears and CB) in subclassification of lung carcinomas on E BUS-TBNA samples & to assess the value of addition of limited panel ICC as an adjunct to cytomorphology in this subtyping.

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1. Introduction

Lung cancer is the leading cause of cancer mortality worldwide.[1] Now a days, EBUS-TBNA has emerged as an accurate tool to evaluate mediastinal lymph nodes and lung parenchymal masses suspected for lung cancer.[2,3] Also, emergence of targeted therapies like epidermal growth factor receptor/tyrosine kinase inhibitors-geftinib, anti vascular endothelial growth factor agents like

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bevacizumab require distinction of NSCC into ADC and SqCC.[4,5] Subtyping of lung carcinoma on cytomorphology is challenging due to overlapping features or poorly differentiated nature of tumor.[6,7] ICC as an adjunctive tool to cytomorphology is available in some studies in literature.[8,9] However, data using this on EBUS-TBNA material is limited with no study reported from India so far.[10–13] As cytology material may be conserved for molecular studies, a limited but optimal use of ICC needs to be determined.[12]

The aim of this study was to know the efficacy of cytomorphology (smears and CB) in subclassification of lung carcinomas on EBUS-TBNA samples & to assess the value of addition of limited panel ICC as an adjunct to cytomorphology in this subtyping.

2. Materials and Methods

The study was conducted on 142 lung car cinoma cases received in d epartment of cytopathology over a period of two and half years.77 cases were studied prospectively and 65 cases retrospectively. All cases satisfied inclusion criteria and were diagnosed as lung carcinoma on EBUS-TBNA specimens on cytomorphology in correlation with clinical and radiological features. All non pulmonary metastatic tumors and tumors other than carcinomas were excluded.

EBUS-TBNA was performed on lung lesions and/or mediastinal lymph nodes, clinically and radiologically suspicious for a malignancy by trained physicians under conscious sedation. Real time US guidance linear EBUS with 22-G aspiration needle was used and two-three passes were made. Both w et fixed and air dried smears were prepared and stained with May Grunwald Giemsa (MGG) and Papanicolaou (Pap) stains respectively. Material for cell block was obtained in same sitting where possible and stained with hematoxylin and eosin (H&E).[14] All cases were cytomorphologically analysed first on smears and subtyped as per WHO (2004) standard description and classification of lung tumors.[6] Cytomorphological fea tures analysed were cellularity(low/high), architecture(acini, sheets, groups, single), cellular features (size, shape), nuclear features (pleomorphism, size, shape, moulding, chromatin, nucleoli), cytoplasm(amount, intracytoplasmic vacuoles, mucin, keratinisation), mitotic activity (zero, occasoinal, numerous) and background (necrosis, apoptotic debri, mucin, inflammation, others). The cases were first grouped into categories of SCC and NSCC. The NSCC were further subtyped into SqCC, ADC, NSCC favouring Sq CC, NSC C favouring ADC, adenosquamous carcinoma (ASC) and NSCC- NOS (not otherwise specified) / poorly differentiated large cell carcinoma (PDLCC).

The same cytomorphological features were then studied on available CB blindly and reported. Then, ICC was applied on cell blocks/adequately cellular smears where available. The primary anti bodies used were TTF1 (Mouse monoclonal antibody, clone- 8G7G3/1, Dako, 1:50 dilution), p63 (Mouse monoclonal antibody, clone-4A4, Bio SB, 1:100 dilution) and synaptophysin (Mouse mo noclonal antibody,clone -snp88, Biogenex, 1:50 dilution). A panel of TTF1 /p63 was selected for NSCC and synaptophysin for SCC /neuroendocrine tumor on morphology.[15–18] Napsin A (Mouse monoclonal antibody, clone napsa, Thermoscientific, 1:600 dilution) was used as secondary antibody in TTF1/p63 negative cases.[19] Immunostaining was done by X Biogenex fully automated stainer operator. Ideally prepared sections from known case of pulmonary carcinoid, SqCC, adenocarcinoma and non neoplastic lung tissue s were used as positive controls for synaptophysin, p63, TTF1 and napsin A respectively. Scoring of nuclear staining with TTF1 and p63 was done by recording percentage of reactive tumour cells[16] (300-500 tumor cells counted) and labeled as 0: 0% of tumor cells staining, 1+: 1-10% of tumor cells staining, 2+:11-49% of tumor cells staining, 3+: $\geq$50% of tumor cells staining (diffusely positive). Pattern 0, 1+ were taken as negative and patters 2+& 3+ as positive result.

Diffuse p63 positivity (3+) and TTF1 negative profile was taken as supportive of SqCC. TTF1 staining (2+, 3+) with p63 negative or focal p63 ($<$50% of tumor cells staining) was taken as supportive of ADC. TTF1 (2+/3+) and p6 (3+) positivity in two different population of cells was suggestive of ASC or else taken as indet erminate. NSCC-NOS was given when both TTF1 and p63 were negative. NapsinA was applied on TTF1/p63 negative profile and taken as positive when$>$10% tumor cells showed cytoplasmic granular staining.[20] Combined cytomorphological diagnosis (smears+cell blocks) was correlated with ICC results. All cases then correlated with fol low up as histopathology (small biopsy, resected specimens), imaging post treatment or other cytopathology specimens (BAL, fluid cytology), where available.

Descriptive stastistics was conducted with statistical package for social science system version SPSS17.0 and categorical variables were expressed as frequencies and percentages.

3. Observation and Results

117of 142cases (82.4%) were classified as NSCC and 25 (17.6%) as SCC on cytomorphology on smears based on singly dispersed small sized cells, presence of nuclear moulding, fine granular chromatin and necrosis in background in SCC cases (Figure 1 a). In 27 of 117(23%) NSCC cases, ADC was made on cytomorphology on smears due to definite presence of intracellular mucin, acinar arrangement, vesicular nuclei, prominent nucleoli and lacy moderate amount of cytoplasm (Figure 1b). 9 of 117cases (7.7%) were made as SqCC due to definite presence of keratinized cells, spindled cells, hyperchromatic nuclei and necrosis (Figure 1c). 21of 117 cases (17.9%)

were categorised as NSCC favouring ADC due to presence of focal acini formation, cells with vesicular nuclei and prominent nucleoli but no mucin (Figure 1d). 11 of 117cases (9.4%) were categorised as NSCC favouring SqCC due to presence of cells in large sheets having hyperchromatic nuclei, levander blue cytoplasm and focal spindling of cells but no keratinisation(Figure 1e). 2 cases were subtyped as NSCC favouring ASC due to presence of mucin and keratinisation in two different population of cells. 39 of 117(33%) cases didn't show any cytomorphological feature of squamous o r glandular differentiation and 8cases were suggestive of PDLCC (Figure 1f). Therefore, total 47of 117 cases (40.2%) could not be subtyped and categorised as NSCC-NOS. Hence, in NSCC group, definite subtyping into ADC or SqCC was possible in 36 of 117 cases (30.8%).

CB were available in 113 of 142 cases. Diagnostic material was available in 105 of 113 cell blocks. 8 CB showed only blood and were unsatisfactory. 85of 105 CB were subtyped as NSC C while 20 CB as SCC on morphology (Figure 2 a). In the NSCC group, diagnosis of 9 cases was upgraded on CB. So, 23 cases were subtyped as ADC (27.1%), 9 SqCC(10.6%), 9 NSCC favouring ADC (10.6%), 9 NSCC favouring SqCC (10.6%), 2 favouring ASC (2.3%) and 33 cases remained as NSC C-NOS (38.8%) (Figure 2b-f). Table 1 shows comparison between cytomorphological diagnosis on FNA smears and CB. Table 2 shows final cytomorphological diagnosis (smears and CB).

Therefore, on cytomorphology alone, a definitive subtyping of NSCC into ADC or SqCC was possible only in 41of 117cases (35.1%). 76 of 1 17 cases (64.9%) comprised of diagnosis favouring (but not definitive) in 33of 117 cases (28.2%) and unclassifiable i.e. NSCC- NOS in 43of 117 cases (36.7%).

Overall, definitive subtyping of all lung carcinoma cases on morphology of EBUS-TBNA smears and CB combined could be achieved in 66 of 142 cases (46.4%).

ICC could be done on 106 of 142 cases that included 96 CB and 10 smears where sufficient material was present for ICC (In 9 of total 105 cases of CB, material was lost). Synaptophysin could be applied on 20 cases of SCC that showed positivity in all cases (Figure 3). A limited panel ICC p63/TTF1 could be applied to 86 cases of NSCC. ICC could be applied on 30of 41 definitive cases on cytomorphology, 22of 33 cases of favouring a diagnosis and 34of 43 cases of NOS on cytomorphology (Figure 4 a-f). Scoring and different patterns of p63 and TTF1 in 86 cases of NSCC is shown in Table 3 a and Table 3b respectively.

Comparing cytomorphological & cytological diagnosis after ICC in these 86 NSCC cases, it is observed that, 68 of 86 cases (79.1%) was given a definitive subtype after ICC with p63/TTF1 as compared to 30of 86(34.9%)cases on morphology and only 18 of 86 cases (20.9%) now remained as unclassifiable as compared to 56 of 86(65.1%) cases on morphology (Table 4). NapsinA was applied on 18o f 86 cases where TTF1/p63 panel was negative (17 cases) or indeterminate (1case). 9 of 18 cases showed positivity for napsin A, therefore, classified as ADC (Figure 5). Now, 9of 86 cases (10.5%) remained as NSCC-NOS. One NOS case on cytomorphology showed large tumor cells dispersed singly, having binucleate plasmacytoid morphology was TTF1, p63 and napsin A negative. However, it showed synaptophysin positivity and classified as large cell neuroendocrine carcinoma (LCNC) (figure6a-6f). Now, only 8 of 86 cases (9.3%) remained as NSCC-NOS.

Overall, 17of 142 cases (11.9%) remained as NSCC-NOS after ICC (TTF1, p63, synaptophysin, napsin A)which was 36.7% on cytomorphology alone. The final cytologic diagnosis (cytomorphology+immunocytochemistry) is shown in Table 5.

Follow up was available in 60 of 142 cases, 59 of which showed concordant diagnosis. In 1 case, cytologic diagnosis was ADC [TTF (3+), p63(1+)]. However, on lung biopsy, it was diagnosed as poorly differentiated SqCC based on focal p63 positivity, but TTF1 was not done on biopsy.

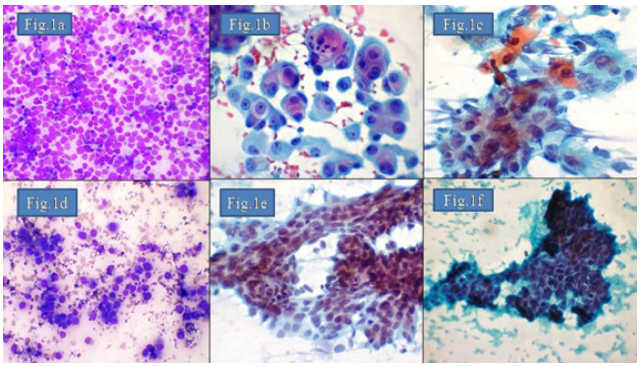

Fig. 1: a: Monomorphic tumor cells in SCC (MGG20X). **1b**: Cells with intracytoplasmic mucin in ADC (Pap 40X). **1c**: Keratinised cells in SqCC (Pap 40X). **1d**: Small groups of tumor cells with vesicular nuclei in case of favouring ADC (MGG 20X). **1e**: Focal spindling of tumor cells with hyperchromatic nuclei in case of favouring SqCC (Pap 20X). **1f**: Tumor cells in patternless sheets in NSCC-NOS (Pap 20X)

Table 1: Comparison of cytomorphological diagnosis on EBUS-TBNA smears and cell blocks.

Smear diagnosis (n)	No. of CB	Diagnosis on CB same as on smears	Diagnosis on CB upgraded from smears	Unsatisfactory
ADC (27)	21	19	0	2
SqCC (9)	9	8	0	1
NSC C favouring ADC (21)	12	7	4 (upgraded to ADC)	1
NSC C favouring SqCC (11)	10	7	1 (upg raded to SqCC)	2
NSC C favouring ASC (2)	2	2	0	0
NSCC-NOS (47)	39	33	4 (2 upgraded to NSCC favouring ADC, 2 upgraded to NSC C favouring SqCC)	2
SCC (25)	20	20	0	0
Total	113			

Table 2: Final cytomorphological subtyping of NSCC (smears+ CB)

Cytomorphological diagnosis (smears+ CB)	No.	%
ADC	31	26.50%
SqCC	10	8.55%
NSC C favouring ADC	19	16.24%
NSC C favouring SqCC	12	10.26%
NSC C favouring ASC	2	1.70%
NSCC-NOS	43	36.75%
Total	117	100%

Table 3: a. Scoring of p63 and TTF1 in NSCC cases (n=86)

Scoring	P63	TTF1
Positive (3+)	17	44
Positive (2+)	4	8
Negative (0,1+)	65	34
Total	86	86

3 b: Patterns of staining with TTF1/ p63 panel in NSCC cases (n=86)

Patterns	No. of cases	Diagnosis
TTF1+/p63 -	48	ADC
TTF1+ /p63+ (focal)	3	ADC
TTF1- /p63 (3+)	16	SqCC
TTF1- /p63 -	17	NSCC-NOS
TTF1++/p63 +++	1	Suggestive of ASC
TTF1- /p63+ (17%)	1	Indeterminate staining (NSCC-NOS)
Total	86	

4. Discussion

Lung cancer contributes a major part to the worldwide cancer mortality.[1] Now a days, due to emergence of targeted therapies and EBUS-TBNA technique, it is possible to manage the patients of lung carcinoma on small cytology samples even when they present at an advanced unresectable stage.[2,3,11–13]

Various studies have shown the utility of EBUS-TBNA over previous techniques.[2,3] However, data regarding its utility in subtyping of lung carcinomas with application of ICC is lacking. Our study could help in highlighting its efficacy in subclassification of lung carcinomas on morphology and using ICC.

In our study, distinction of 142 cases into 25 SCC and 117 cases of NSCC could be made on cytomorphology based on above mentioned characteristic features.[20,21] We tried to classify 117 cases of NSCC into definite ADC and SQCC on smears and CB based on definite features like intracellular mucin and keratinisation in case of ADC and SQCC respectively.[20] On smears only 30.8% cases were given definitive diagnosis and 69.2% remained either as NOS(40.2%) or given a diagnosis favouring (29%) but not definitive. CB could help in upgrading diagnosis in 9of 105

Table 4: Comparison of cytomorphological diagnosis with results of ICC panel (TTF1, p63, synaptophysin)

Cytomorphological subtype	No. (smears+ cell bocks)	Cases where IHC done(n)	Same subtyping as on cytomorphology after ICC (n)	Different subtype/ upgradation after ICC
ADC	31	22	20	2 (NOS)
SqCC	10	8	8	0
NSC C favouring ADC	19	11	0	7 (ADC), 4 (NOS)
NSC C favouring SqCC	12	9	0	3 (ADC), 5 (SqCC), 1 (NOS)
NSC C favouring ASC	2	2	0	1(ADC) 1 (SqCC)
NSCC-NOS	43	34	10	20 (ADC), 2 (SqCC), 1(suggestive of ASC), 1 (LSNC)
SCC	25	20	20	
Total	142	106	58	48

Table 5: Final cytologic subtyping of lung carcinoma on EBUS- TBNA samples (cytomorphology + ICC)

Subtype	No. of cases subtyped after ICC	Cases diagnosed on cytomorphology without ICC	Total no. of cases	Percentage (%)
ADC	60	9	69	48.59
SqCC	16	2	18	12.67
NSCLC favouring A DC	0	8	8	5.63
NSCLC favouring SqCC	0	3	3	2.14
NSCLC suggestive of ASC	1	0	1	0.7
LSNC	1	0	1	0.7
NSCLC-NOS	8	9	17	11.97
SCC	20	5	25	17.6
Total	106	36	142	100

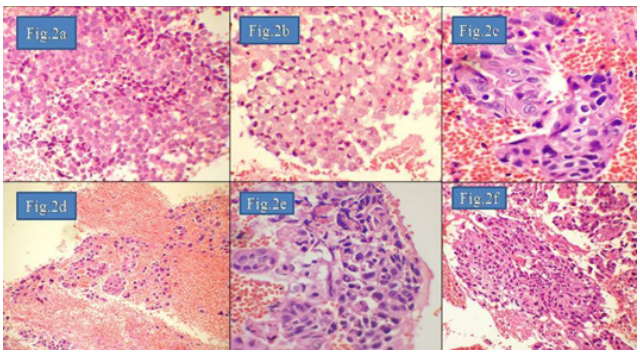

Fig. 2: **a**: Cell block, SCC (H&E 20X). **2b**: ADC with intracytoplasmic mucin (H&E 20X). **2c**: SqCC showing keratinisation (H&E 40X). **2d**: Case of favouring ADC (H&E 20X). **2e**: Case of favouring SqCC (H&E 40X). **2f**: NSCC-NOS (H&E 10X).

available CB with diagnostic material. In our study, it was observed that architectural features were better appreciated on CB, while nuclear and cytoplasmic details were better seen on FNA smears. Therefore, FNA smears and CB in this study were complementary to each other in defining morphological features. On evaluating cytomorphology alone (smears+CB), 41of 117 NSCC cases (35.1%) could be given a definite subtype of ADC (31 cases) or SqCC(10

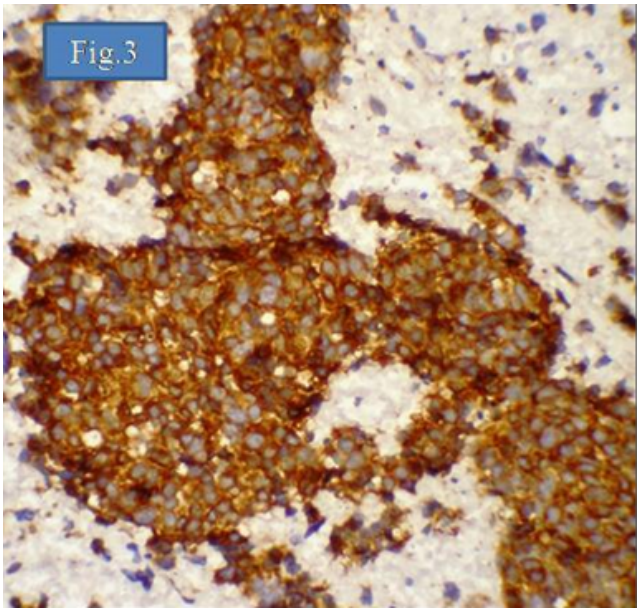

Fig. 3: SCC showing cytoplasmic positivity (synaptophysin stain 20X).

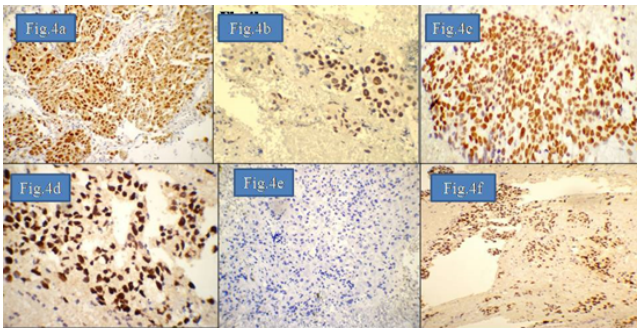

Fig. 4: a: Diffuse nuclear staining in SqCC (p63 stain 10X). **4b**: Focal nuclear staining in case of favouring SqCC (p63 stain 20X). **4c**: Diffuse nuclear staining ADC (TTF1 stain 20X). **4d**: Focal staining in case of favouring ADC (TTF1 stain 20X). **4e**: Negative staining in case of NSCC-NOS (p63 stain 10X). **4f**: Focal positivity in the same case of NSCC-NOS (TTF1 stain 10X).

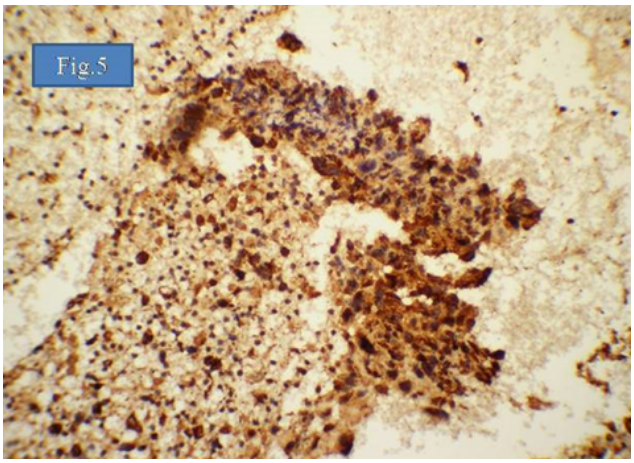

Fig. 5: Cytoplasmic granular staining inTTF1/p63 negative case (napsin A stain 20X).

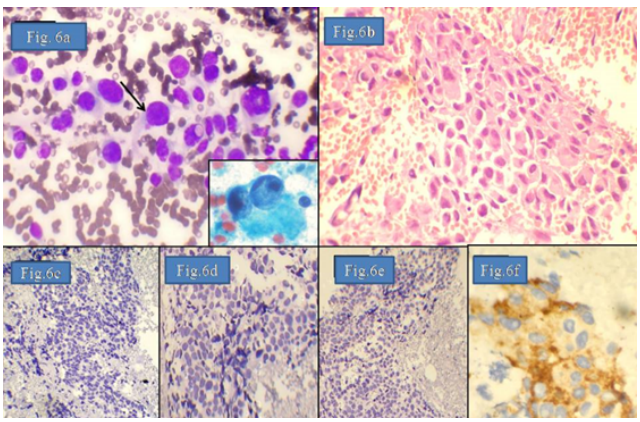

Fig. 6: a: Singly dispersed tumor cells (MGG 40X). Inset shows binucleate plasmacytoid cells with prominent nucleoli (Pap 40X). **6b**: Cell block (H&E 40X). **6c**: Negative staining (TTF1 stain 10X). **6d**: Negative staining (p63 stain 20X). **6e**: Negative staining (napsin A 10X). **6f**: Cytoplasmic positivity (synaptophysin stain 40X) .

cases). Other 33 of 117NSCLC cases (28.2%) were given a diagnosis favouring but not definite for a subtype. 43 of 117cases (36.7%) remained as NSCC- NOS. Overall, on cytomorphol ogical subtyping of 142 lung carcinoma cases on EBUS-TBNA smears and CB together, 66 of 142 cases could be definitely classified that included 25 SCC and 41 NSCC cases. Previous studies have shown that on small specimens, only 47% to 78% is classifiable into specific subtype on morphology alone and unclassifiable (NSCC NOS) accounts for 22% to 53% cases.[8,22] Further subtyping is not possible on morphology alone due to absence of specific features or presence of overlapping features.[6,7] Therefore, to decrease NSCC-NOS cases, preparation of CB and application of ICC is required. Nicholson et al[23] in their study of EUS and EBUS-TBNA samples of NS CC-NOS cases have shown the utility of ICC in refining the diagnosis of NSCLC-NOS to either SQCC or ADC.

Most of the previous studies used 3-9 antibodies including p63 and TTF-1 antibodies.[16–18,23–25] Since cytology material is limited and needs to be conserved for molecular studies, limited but optimal use of ICC needs to be done.[24]

Mukhopadhyay and Katzenstein[25] (applied 6 ICC markers) and Pelosi et al[26] (applied 5 ICC markers) on NSCC biopsies and classified 77% and 94% of the cases respectively. In cytology samples also, ICC brought down NOS cases to 4% -14%.[27,28] However, only few studies have applied immunostains for subtyping of lung cancer on EBUS-TBNA samples.[10–12]

Table 6 shows previous studies that have used immuno-histochemical markers as an adjunct to cytomorphology to refine lung carcinoma subtypes on small and large samples.

5. Result

In our study, synaptophysin was applied in 20 of 25 SCC cases diagnosed on cytomorphology. It helped in confirming the diagnosis in all SCC cases. In NSCC group, a panel of TTF1/p63 immunostain was applied on 30 of 41 cases with definitive morphology, all these cases showed complete corre lation with ICC results and follow up where available highlighting the specific nature of morphological observations when present. In 22 of 33 cases with diagnosis favouring a subtype on morphology, ICC with TTF1/ p63 upgraded 17 of 22 cases (77.3%) to a definitive diagnosis. Interestingly, three cases with diagnosis of favouring SqCC on morphology showed ICC pattern compatible with ADC (TTF1 3+/ p63-) which on follow up were also ADC in two cases. One case had no follow up. This indicates that this diagnosis on morphology may not be very accurate and should not be loosely used. It would be best to apply ICC in such cases. In 43 cases of NSCC-NOS on morphology, ICC was done in 34 cases that helped to arrive at definitive diagnosis in 23 cases.

Table 6: Studies with immunomarkers as an ancillary technique:

S.No.	Researcher	Immunostains used	Size and type of sample	Result
1.	Zhang H et al[7]	TTF1, p63, high molecular weight keratin, p16	Bronchoscopic biopsies (13 cases) & surgical specimens (47 cases).	TTF1, p63, HMWK,p16(INK4A) is effective for distinguishing between SCC and poorly SqCC.
2.	Sinna EA et al[8]	TTF1, p63	40 cases of guided FNAC of lung lesions	TTF upgraded sensitivity of ADC from 83.3% on cytomorphology to 87.5%.P63 increased sensitivity of 91% on cytomorphology to 94.7% in case of SqCC.
3.	Wu M et al[9]	TTF1, p63	30 cytology cases of primary and metastatic lung cancer	All lung ADC were TTF1+ and all primary lung SqCC were p63+.
4.	Navani N et al[10]	TTF1, p63, Cytokeratin5/6	774 EBUS-TBNA samples of suspected lung cancer	77% cases could be subtyped accurately using ICC.
5.	Wallace WAH et al[11]	ICC using TTF1	48 EBUS/EUS-FNA samples of lung cancer	Accuracy subclassify 37 out of 48 cases.
6.	Liu A et al[12]	IHC used	55 EBUS-TBNA samples of mediastinal mass	Sensitivity and diagnostic accuracy of EBUS-TBNA in diagnosis of lung cancer were 92.5% and 94.5%respectively and IHC help in confirming 37 of 55 cases.
7.	Kasprzak A et al[15]	chromogranin A, neuron-specific enolase, synaptophysin, protein gene product 9.5	Histology samples of pulmonary tumors	Synaptophysin is the most specific marker of neuroendocrine differentiation
8.	Kimbrell et al[16]	CK7, CK5/6, TTF-1 and p63	140 cytology cases of lung tumors	56% were classified by cytomorphology alone. TTF1 classified 89% of ADC and p63 classified 100% of SqCC.
9.	Terry J et al[17]	p63, TTF1, CK5/6, CK7, 34βE12, Napsin A, mucicarmine, NTRK1, and NTRK2	200 cases of ADC and 225 SqCC in tissue microarray.	p63 is best marker to separate ADC from SCC with sensitivity 84%, specificity 85%).
10.	Ao MH et al[18]	TTF1,napsin A, p40	200 lung cancer tissue microarrays	In ADC sensitivity and specificity of triple marker were 93.5% and 77.5%, respectively.
11.	Stoll ML et al[19]	TTF1, napsin A	75 cytolgy samples of pulmonary carcinomas	Sensitivity and specificity of TTF-1 were 81%, napsin-A had specificity of 96%, and sensitivity of 65% for lung ADC.
12.	Nicholson A et al[23]	Mucin, TTF-1, cytokeratin 5/6, and p63	32 NSCLC cases of small biopsies and cytologic material	Refinement of diagnosis in 65% cases of NSCLC to either SQCC or ADC
13.	Mukhopadhyay S et al[25]	TTF-1, napsin A, p63, and CK5/6.	39 small biopsies and resected specimen	TTF-1, napsin A, p63, and CK5/6 classified 30 of39 (77%) cases accurately.
14.	Pelosi G et al[26]	cytokeratins 5/6 and 7, p63, TTF-1,vimentin	63 biopsies and surgical resected specimens	59 of 63 (94%) lesions were correctly classified compared with 53 of 63 (84%) on morphology,
15.	Righi L et al[27]	cytokeratin 7, CK5, TTF1, p63, p40, napsin A, desmocollin-3	103 FNAC samples with morphological diagnosis of NSCLC-NOS	NSCLC-NOS decreased from 36% to 14% after IHC.

Hence, in these 86 cases of NSCC with ICC, comparing cytomorphology results with limited panel TTF1/p63 ICC results showed that 34.9% of NSCC cases could be given a definitive subtype on cytomorphology alone which increased to 79.1%(68 of 86 cases) & the unclassifiable cases could be brought down to 20.9% (18 of 86 cases) after limited panel ICC. Our results were comparable to results of previous studies with immunostains TTF/P63 on small specimens[10,11] Addition of napsin A as a secondary antibody further reduced NOS cases from 18 of 86 (20.9%) after 1st panel ICC to 9 of 86(10.5%). It was observed that staining with napsinA gave more background staining than crisp nuclear staining of TTF1. Follow up was available in 3 of 9 cases that showed concordant result. In our study napsin A could add on to the definitive cases of ADC in TTF1 negative cases. In the remaining 9 NOS cases, one case was PDLCC was suspicious for neuroendocrine tumor on morphology was TTF1-/p6 3-/napsin A negative & was diagnosed as LCNC based on synaptophysin positivity. Follow up was available in 2 of the remaining 8 NOS (TTF1, p63, napsin A negative) cases, one was diagnosed as ADC on transbronchial lung biopsy while other was poorly differentiated carcinoma on biopsy. So, synaptophysin contributed 1case to diagnose LCNC. After total ICC in 86 cases, subtyping could be achieved in 90.7% of NSCC cases with only 9.3% remaining as unclassifiable/NSCC-NOS.

Overall, 17 of 142 cases (11.9%) remained as NSCC-NOS using all synaptophysin, TTF1 and p63 antibodies as 1st panel and napsin A as secondary antibody. So, in our study, we could give definite diagnosis in 88.1% cases. This value is comparable to Wallace and Rassl[11] study who could classify in 84% cases using ICC. Hence, this study brings out the significant role of ICC as an adjunct to cytomorphology on EBUS-TBNA specimens and highlights that limited panel ICC (p63, TTF1, synaptophysin with napsin A in only subset of cases) is sufficient to classify in majority (90%) of cases that also allows material to be preserved for further molecular studies.

6. Conclusion

This study concluded that subtyping of lung carcinoma on EBUS-TBNA samples is feasible. Preparation of cell blocks adds to cytomorphological diagnosis and provides material for ICC. Use of limited panel ICC comprising of TTF1/p63/ synaptophysin as an adjunct to cytomorphology significantly increases the proportion of classifiable cases and is sufficient in majority of the cases. So, it is recommended that centres performing EBUS- TBNA in suspected lung cancer cases should prepare cell blocks in addition to smears, and incorporate limited panel ICC for correct typing of lung carcinomas.

7. Source of funding

None

8. Conflicts of interests

None

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