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CLASSIFICATION OF LUNG TUMOURS BY IMMUNOCYTOMORPHOLOGY ON LIQUID BASED CYTOLOGY

Pathology

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ABSTRACT

Introduction: Lung cancer accounting for 13% of all new cancer cases worldwide is the commonest cancer in men in India. Liquid Based Cytology, a newer automated cytology sample processing technique which has well proved its efficiency in handling gynecology samples is now emerging as a promising tool in processing of non-gynecology samples. Research question in the study here is that "Can the new technique of Liquid based Cytology with immune markers be used in processing small specimen obtained by endobronchial ultrasound guided- transbronchial needle aspiration to classify lung tumours?".

Material and Methods: 35 primary lung cancer cases were included in the study. Conventional (giemsa and or pap stained smears) and LBC slides (1 stained and 3 unstained) were prepared from EBUS-TBNA samples. p63, TTF1 and chromogranin immune markers were applied on the unstained slides. Immunocytochemical staining for all three immune markers were interpreted and tumours were classified into histological subtypes. Cytomorphological features were studied on conventional smear and LBC stained smears.

Results: Out of 35 cases (100%), 10 (28.57%) were scant in cell population on conventional cytology smears while only 4 (11.43%) on LBC. On basis of positive immunocytochemistry (p63, TTF1 and chromogranin), 10 (28.57%) cases were diagnosed as NSCLC favour Adenocarcinoma, 10 as NSCLC favour SCC, 1 (2.86%) as NSCLC-NOS, 3 (8.57%) as squamous cell carcinoma and 7 (20%) as small cell carcinoma. All the cases were correlated with histopathological diagnosis confirmed with immunohistochemistry (IHC).

Conclusion: The sensitivity of LBC SurePath (88.57%) was higher than conventional smears (71.43%). LBC produces desired number of slides making use of the ancillary techniques, including immunocytochemistry, molecular analysis and in situ hybridization possible. There were certain morphological changes which can be overcome by standardization of technique and short duration staff training.

KEYWORDS

Liquid Based Cytology, Lung Tumours, Immunocytochemistry.

INTRODUCTION

Lung cancer is one of the commonest cancers and cause of cancer related deaths worldwide. It accounts for 13% of all new cancer cases and 19% of cancer related deaths all over world.[1] In India lung cancer constitutes 6.9% of all new cancer cases and 9.3% of all cancer related deaths in both sexes, it is the commonest cancer in men with the highest reported incidence from Mizoram in both males and females.[2] Male to female ratio is 3.5:1. Cancer lung occurs most often between ages of 40 and 70 years, only 2% of lung cancers appear before age of 40 years.[3]

Lung cancers have been classified according to histological type. The previous 1967, 1981 and 1999 WHO classifications addressed lung cancer classification based mainly on resection specimens. Inclusion of cytology was done for the first time in 2004 WHO classification.[4,5,6] The 2015 World Health Organization (WHO) classification of lung tumours is the foundation for lung cancer classification. In contrast to previous classification systems, the 2015 WHO classification relies to a greater extent on immunohistochemical characterization in addition to light microscopy, allowing for subtyping that more judiciously guides treatment strategy and predicts clinical course. Also, it provides standardized criteria and terminology for lung cancer diagnosis on small biopsies and cytology, which is critical, given that the majority of patients with lung cancer present with advanced stage disease and are not surgical candidates. Most tumours can be classified using a single adenocarcinoma marker (e.g., TTF-1 or mucin) and a single squamous marker (e.g., p40 or p63). For neuroendocrine differentiation chromogranin, synaptophysin and CD56 are widely used.

Liquid based cytology (LBC), a newer cytopreparatory technique has been designed to provide better cell preservation and improved quality smears. Two types of LBC systems are available- SurePath and ThinPrep. The utility of TP of various types of lung samples like sputum, bronchial washings, or lavage has been widely accepted as a major diagnostic and has been proven to have a great association with tissue histological diagnosis.[7] Over conventional cytology this method has various advantages including better fixation, uniform thickness smears, no drying artifact and removal of obscuring blood and mucous.[8]

Endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) has emerged as an accurate and sensitive tool for the diagnosis and staging of lung cancer.[9] As this is an invasive procedure, repeat procedure is difficult, also a lot of times sample yield is small. Hence it becomes very essential to search for better cytological preparations to give accurate results. So the LBC may prove an appropriate tool to process these small specimens obtained by EBUS-TBNA. Also utilizing the advantage of unstained prepared slides, immunocytochemistry markers can be applied for definite histological subtyping.

OBJECTIVES-

1. To evaluate immune markers: P63, TTF-1 and chromogranin on LBC slides.
2. To classify the lung cancer on LBC by immunocytomorphology.
3. To study cytomorphology of lung carcinoma on liquid based cytology and conventional cytology.

MATERIALS & METHODS:

The study was a tertiary health care institute based prospective cross-sectional study with a sample size of 35 cases carried out for a duration of 20 months. The study was conducted after obtaining permission from the institutional ethical committee.

Inclusion Criteria:

1. Clinically and bronchoscopy suspected cases of lung cancer
2. Fresh cases without prior radiotherapy or chemotherapy.

Exclusion Criteria:

1. Patients on chemotherapy were excluded from the study.
2. Patients unable to undergo procedure of ultrasound guided endoscopic transbronchial aspiration.

The patients included were classified as per the guidelines for small lung biopsies and cytology laid down by 2015 World Health Organization (WHO).[10]

Routine clinical details and investigations were taken from clinical case sheet for following specific details:

- Age
- Duration
- Radiological findings

Specimen collection: Samples were obtained by endobronchial ultrasound-guided transbronchial needle aspiration using flexible probe done under procedural sedation and local anaesthesia performed on supine position with operator standing on head end of bed.

METHOD:

1. Samples were obtained by endobronchial ultrasound-guided transbronchial needle aspiration aspiration was done by Olympus bronchoscope where 21 G needle used after localization of the mass lesion. Three passes were done in each case.
2. Conventional smears were made immediately after aspiration on the site of the procedure, while approximately half of the aspirate was preserved in CytorichRed preservative and sent to the laboratory for processing.
3. Conventional smears were stained with Giemsa and also Pap if enough material was available.
4. Slides were prepared by LBC technique (SurePath). One Pap stained slide and three unstained slides were prepared.
5. Immune markers were applied on all three LBC prepared unstained slides. slides were kept immediately in cooled acetone. Primary antibodies were put on the slides, covered with coverslip and kept in humid chamber in a refrigerator overnight to maintain the chamber at 4□C. After that slides were taken out and washed with tris buffer. Secondary HRP primer was added for 30 minutes followed by washing in tris buffer again. Haematoxylin was used as counterstain for 10-15 seconds again washed in tris buffer and mounted with DPX.
6. Cytomorphological features were studied on conventional smear and LBC stained smears.
7. Immunocytochemical staining for p63, TTF1 and chromogranin immune markers were interpreted and tumours were classified into histological subtypes according to 2015 WHO classification of tumours of lung.
8. Cytological diagnosis was confirmed with histopathological diagnosis in each case.
9. Results were analyzed statistically.

Statistical Analysis-

Categorical variables were presented in number and percentage (%) and continuous variables were presented as mean ± SD and median. Statistical tests were applied as follows-

1. Qualitative variables were correlated using Chi-Square test /Fisher's exact test and Mcnamer test was used to compare sensitivity of conventional cytology and LBC PAP.
2. Diagnostic test was used to find out the sensitivity, specificity, NPV and PPV of conventional cytology, LBC PAP and Diagnosis ICC. A p value of <0.05 was considered statistically significant.

The data was entered in MS EXCEL spreadsheet and analysis was done using Statistical Package for Social Sciences (SPSS) version 21.0.

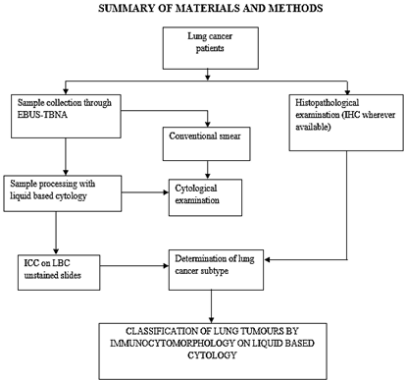

Figure 1 summarizes materials and methods.

RESULTS:

A total of 35 primary lung cancer cases were studied. Age range varied from 44- 75 years with a mean age of 59.63 years. 31 (88.57%) cases were males while only 4 were females (11.43%). On conventional cytology out of 35cases (100%), 22 (62.85%) were positive for tumour cells showing tumour cells on giemsa and or pap stained cytology smears, 4 (8.57%) showed occasional atypical cells on smears, 10 (28.57%) were scant in cell population mainly showing blood clot. On basis of findings on conventional cytology smears, out of thirty five cases, ten (28.57%) were diagnosed as inadequate, twelve (34.29%) non-small cell lung carcinoma (NSCLC), four (11.43%) non-small cell lung carcinoma possibly adenocarcinoma (Figure 2), 2 (5.71%) squamous cell carcinoma, 4 (11.43%) small cell carcinoma (Figure 3) and three (8.57%) suspicious showing occasional atypical cells on smears as shown in Table 1.

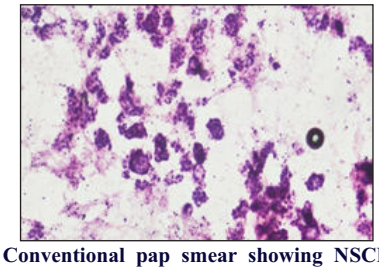

Figure 2: Conventional pap smear showing NSCLC possibly adenocarcinoma

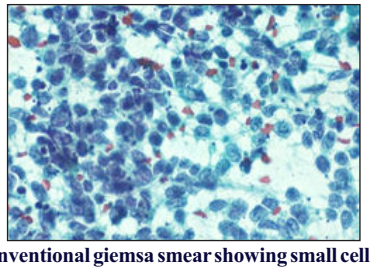

Figure 3: Conventional giemsa smear showing small cell carcinoma

Table 1: Distribution Of Cases According To Diagnosis Conventional

DIAGNOSIS	NO. OF CASES	PERCENTAGE (%)
INADEQUATE	10	28.57
NSCLC	12	34.29
NSCLC POSSIBLY ADENOCARCINOMA	4	11.43
SCC	2	5.71
SMALL CELL CARCINOMA	4	11.43
SUSPICIOUS	3	8.57
TOTAL	35	100.00

On Liquid Based Cytology (LBC), as shown in Table 2 below, 16 (45.71%) cases were classified as NSCLC, 4 (11.43%) as NSCLC possibly adenocarcinoma (Figure 4),1 (2.86%) as NSCLC possibly squamous cell carcinoma, 3 (8.57%) as squamous cell carcinoma and 7 (20%) as small cell carcinoma (Figure 5).

Table 2: Distribution of Cases According to Diagnosis LBC

LBC SMEARS	NO. OF CASES	PERCENTAGE (%)
INADEQUATE	4	11.43
NSCLC	16	45.71
NSCLC POSSIBLY ADENOCARCINOMA	4	11.43
NSCLC POSSIBLY SCC	1	2.86
SCC	3	8.57
SMALL CELL CARCINOMA	7	20.00
TOTAL	35	100.00

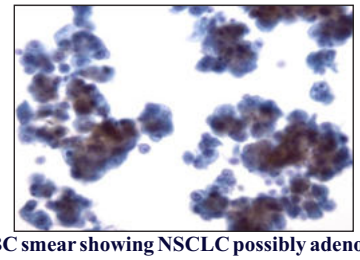

Figure 4: LBC smear showing NSCLC possibly adenocarcinoma

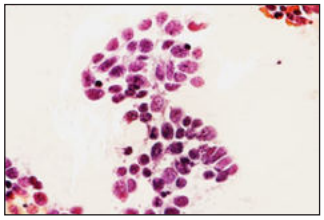

Figure 5: LBC pap smear showing small cell carcinoma

For more definite histological subtyping and confirmation immunocytochemistry for p63, TTF1 and chromogranin was applied on all cases. Distribution of cases according to positive immunocytochemistry is shown in the Table 3.

Table 3: Distribution of Cases according to positive ICC

ICC	NO. OF CASES
p63	13
TTF1	13
CHROMOGANIN	7
INCONCLUSIVE	4

On basis of positive immunocytochemistry cases were diagnosed according 2015 WHO Classification of lung tumours. 10 (28.57%) cases were diagnosed as NSCLC favour Adenocarcinoma, 10 as NSCLC favour SCC, 1 (2.86%) as NSCLC-NOS. 3 (8.57%) cases diagnosed as squamous cell carcinoma and 7 (20%) as small cell carcinoma on LBC are confirmed by immunocytochemistry. Distribution of cases on basis of diagnosis on immunocytochemistry (ICC) is given in the table 4.

Table 4: Distribution of the Cases according to Diagnosis on ICC

DIAGNOSIS	NO. OF CASES	POSITIVE MARKERS	PERCENTAGE (%)
INCONCLUSIVE	4	-	11.43
NSCLC FAVOUR ADENOCARCINOMA	10	TTF1	28.57
NSCLC FAVOUR SCC	10	P63	28.57
NSCLC-NOS	1	-	2.86
SCC (ON MORPHOLOGY)	3	p63	8.57
SMALL CELL CARCINOMA	7	CHROMOGRANIN, (3 cases also TTF 1 +)	20.00
TOTAL	35		100.00

All the cases were correlated with histopathological diagnosis confirmed with immunohistochemistry (IHC). Table 5 lists the histopathological diagnosis of the cases.

Table 5: Distribution of the Cases according to Histology & IHC

DIAGNOSIS	NO. OF CASES	PERCENTAGE (%)
ADENOCARCINOMA	4	11.43
NSCLC COULD BE ADENOSQUAMOUS	2	5.71
NSCLC FAVOUR ADENOCARCINOMA	6	17.14
NSCLC FAVOUR SCC	7	20.00
NSCLC WITH NEUROENDOCRINE MORPHOLOGY POSSIBLY LARGE CELL CARCINOMA	1	2.86
NSCLC-NOS	1	2.86
SCC	7	20.00
SMALL CELL CARCINOMA	7	20.00
TOTAL	35	100.00

According to histopathology and IHC diagnosis following 2015 WHO classification, four were adenocarcinoma could be classified on histopathology only later on confirmed with IHC, 6 were NSCLC favour Adenocarcinoma subtyped with IHC.

2 cases diagnosed as NSCLC favour SCC on LBC and ICC were reclassified as NSCLC could be Adenosquamous on histopathology and IHC. 7 (20%) were diagnosed as squamous cell carcinoma, 7 (20%) as small cell carcinoma, 1 (2.86%) as NSCLC with neuroendocrine differentiation possibly large cell carcinoma and 1 (2.86%) as NSCLC-NOS.

Table 6 and Table 7 shows comparison between LBC pap and conventional cytology.

Table 6: Comparison between Conventional and LBC

		CONVENTIONAL CYTOLOGY		TOTAL
		NEGATIVE	POSITIVE	
LBC PAP	NEGATIVE	4 (11.43%)	0 (0.00%)	4 (11.43%)
	POSITIVE	6 (17.14%)	25 (71.43%)	31 (88.57%)
	TOTAL	10 (28.57%)	25 (71.43%)	35 (100.00%)

Table 7: Distribution of Cases according to Cellularity and Cell morphology

		LBC PAP		Total	P value
		POSITIVE FOR TUMOUR CELLS	SCANT		
CONVENTIONAL CYTOLOGY	ATYPICAL CELLS	3 (9.68%)	0 (0.00%)	3 (8.57%)	0.004
	POSITIVE FOR TUMOUR CELLS	22 (70.97%)	0 (0.00%)	22 (62.86%)	
	SCANT	6 (19.35%)	4 (100.00%)	10 (28.57%)	
Total		31 (100.00%)	4 (100.00%)	35 (100.00%)	

As observed above, 10 cases (28.57%) were inadequate on conventional cytology while only 4 (11.43%) on LBC pap. LBC PAP (Sensitivity- 88.57%) turned out to be better than conventional cytology (Sensitivity- 71.43%).

Diagnosis on LBC and ICC were correlated with diagnosis on histology and IHC. Diagnosis of inconclusive cases could be made on histology with IHC. Out of 4 Inconclusive cases on LBC, 1 was diagnosed as NSCLC favour SCC, 1 as NSCLC with Neuroendocrine morphology possibly Large Cell Carcinoma, 1 as NSCLC-NOS and 1 as SCC. One case diagnosed as NSCLC-NOS turned out to be NSCLC favour SCC. 2 (5.71%) cases diagnosed as NSCLC favour SCC reclassified into NSCLC could be Adenosquamous type.

DISCUSSION:

Thirty-five well known lung cancer cases were included in the study, there were 31 male patients and 4 female patients. This is representative of current Indian scenario.[11] All our cases were in advanced stage i.e. stage III and stage IV at the time of presentation. The most common clinical presentation in our patients was cough followed by weakness, dyspnoea, weight loss, haemoptysis, and chest pain. 51% cases presented with cough. Remaining cases presented with fatigue (n=10, 28.86%), dyspnoea (n=8, 22.86%), weight loss (n=8, 22.9%), haemoptysis (n=6, 17.1%) and chest pain (n=2, 5.7%). LBC is well established for screening and diagnosis of cervical diseases.[4, 12] Recently LBC use in nongynecological specimen processing has risen to improve the diagnostic yield. In our study samples were obtained by EBUS-TBNA using flexible probe. On conventional cytology, smears were assessed for cellularity. Smears showing predominantly haemorrhage or clot and or inflammatory cells obscuring the lesional cells were labelled as scant in cellularity and non-diagnostic. Diagnosis in these cases was given as inadequate for opinion.Ten cases (28.57%) were inadequate. Three cases (8.57%) were showing occasional atypical cells and also few of the cells were obscured by haemorrhage. These cases were labelled as suspicious for malignancy. Twenty-two (62.86%) cases were adequate in cellularity and on basis of cytomorphological features were classified into respective histological subtypes.

Szlubowski et al in 2007, assessed the diagnostic yield of transbronchial needle aspiration (TBNA) in mediastinal or hilar adenopathy in lung cancer and non-malignant lesions. TBNA technique using conventional cytology was diagnostic in 67.1% of lung cancer patients.[13]

Lung cytology has been the first nongynecological application of LBC

as described by Arbyn et al in their article published in 2008.[14] LBC has been utilized in processing of various lung cytology specimens like bronchial washings, bronchial brushings, broncho-alveolar lavage fluid and EBUS-TBNA.

Liu et al[15] and Fan et al[16] evaluated the diagnostic value of LBC using ThinPrep and SurePath bronchial brushing cytology in early diagnosis of lung cancer. Fan et al reported that LBC has obviously higher diagnostic sensitivity for the detection of central lesions (78.6%) than the conventional smear method (60.3%, $p < 0.01$). This is in accordance with our study. Out of thirty-five cases, thirty-one were positive for tumour cells and could be given a definite diagnosis. The sensitivity of SurePath liquid-based preparations (88.57%) was higher than conventional smears (71.43%). The difference between the two groups was significant ($p < 0.05$). The higher adequacy rate of LBC smears in comparison to conventional smears can be explained by many factors. LBC offers several advantages, including the ability to transport in a stable collecting media, removal of blood clot, necrotic debris, absence of drying artifacts and mucous.[17] Though, this also leads to slight reduction in cellularity of LBC smears. With LBC system we were available with limited number of slides to screen this led to ease in screening with increased efficiency. And also, we were available with the option to apply ancillary techniques on additional preparations. Like in our study also we prepared extra unstained slides for application of immunocytochemistry to do a more specific histological subtyping of cases.

There were certain changes "morphological and architectural" in LBC smears compared to conventional smears as reported by Kobayashi et al.[18] RBCs are lysed by CytoRich red preservative and decanted except in very haemorrhagic specimens where few ghosts of RBCs may be left in smears. Necrotic debris become difficult to analyse. Large aggregates and clusters of cells seen on conventional smears were fragmented in LBC smears and appeared as small clusters and cell aggregates. Cells appeared smaller in LBC smears than conventional smears. Chromatin details, nuclear pleomorphism and membrane irregularity were well preserved in LBC smears. Nucleoli were more prominent.

Immunocytochemistry using a panel of p63, TTF1 and chromogranin was applied on LBC unstained slides. Sixteen (45.71%) cases, which were classified as NSCLC only on LBC pap could be subtyped further into NSCLC favour Adenocarcinoma, NSCLC favour SCC and NSCLC-NOS. Various studies are available in literature classifying lung carcinoma cases using limited immune markers panel. Righi et al[19] used a panel of 4 antibody panel including cytokeratin (CK) 7, CK5, TTF1 and p63 on FNA specimen from 103 NSCLC-NOS cases. Collins et al[20] used panel of Napsin-A, TTF-1, CK5/6, and p63 on 81 EBUS FNA specimens from primary pulmonary non-small cell carcinoma cases and could classify 85% cases.

TTF1 (Figure 6) was positive in all 10 cases of adenocarcinoma.

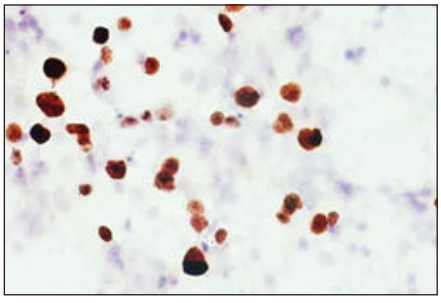

Figure 6: LBC ICC showing TTF1 positivity

Inadequate cases were excluded during calculation of sensitivity and specificity of TTF1 immunocytochemistry as smears of inadequate cases were scant in cellularity and no interpretation was possible. Also 2 NSCLC possibly adenosquamous carcinoma cases were excluded considering the possibility that aspiration may have been done from areas of squamous differentiation only. Three small cell carcinoma cases were positive for TTF1. Thus, in our study TTF1 showed 100% sensitivity and 87.5% specificity. Argon et al21 described 100% sensitivity and specificity for TTF1. P63 (Figure 7) was positive in 13 cases, out of which 2 turned out to be NSCLC possibly adenosquamous carcinomas on IHC.

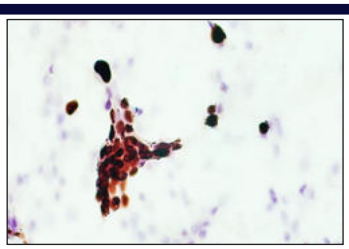

Figure 7: LBC ICC showing p63 positivity

1 case was missed on immunocytochemistry which was picked on IHC. 2 inconclusive cases were diagnosed on IHC, 1 as SCC and other as NSCLC favour SCC. Excluding inadequate cases and NSCLC favour adenosquamous carcinoma cases p63 was 91.67% sensitive and 100 % specific, also Wu et al[22] reported 100 % sensitivity and 100 % specificity for p63. Chromogranin was positive all seven cases of small cell carcinoma and showed 83.33% sensitivity and 100% specificity. Roy et al[23] reported expression of any two of three neuroendocrine markers (CD56, Synaptophysin, Chromogranin-A) in 100% of the cases.

Thus, we summarize LBC as a better system for lung cancer cytology and addition of immunocytochemistry may turn into an invaluable tool to subtype lung carcinoma cases.

CONCLUSION:

We found that LBC is a reliable diagnostic tool in lung cancer cytology to utilize specimen from endobronchial ultrasound guided transbronchial needle aspiration. It improved the adequacy rate as well as provided a more definitive histological subtyping instead of a broad categorization. However, a well standardized and validated immunocytochemical staining protocol must be in place in order to avoid erroneous results from technical errors. The sample size of our study was small. We recommend further studies with lager sample size for standardization and establishment of technique in lung cancer cytology.

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