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Original Article

Limited Immunohistochemistry Panel in Sub-classification of Poorly Differentiated Non-Small Cell Lung Carcinoma in a Scarce Resource Setup

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Abstract:
Background: With the emergence of targeted therapies for non-small cell lung carcinoma with different efficacy and toxicity, differentiating between adenocarcinoma and squamous cell carcinoma in lung biopsies has become essential. In cases of poorly differentiated non-small cell lung carcinoma standard haematoxylin and eosin stained morphology cannot confidently subtype the tumour as adenocarcinoma and squamous cell. In these cases, limited panel of immunohistochemical markers including TTF-1, CK7 and p63 may serve as a useful tool to specify the subtype of lung carcinoma in a resource limited setup.
Objectives: The aim of this study is to see the expression of IHC markers TTF-1, CK7 and p63 in poorly differentiated NSCLC and its importance in subtyping NSCLC as ADC & SCC in lung biopsy specimens.
Methods: It was a Cross sectional study, conducted at Department of Pathology, SZABMU, PIMS Islamabad for a period of 12 months (September 2015 to August 2016).
A total of 51 lung biopsies with poorly differentiated NSCLC were further stained with the IHC markers TTF-1, CK 7 and p63. Statistical analysis was performed using SPSS Version 21.
Results: The mean age was 59±14 years with a male to female ratio of 2:1. 26 males (51%) and 5 females (9.8%) out of the total 51 cases were smokers. 41.2% cases expressed positivity for TTF-1, 39.2% for p63 and 51% were positive for CK7 staining. 80.4% of cases were sub-typed as ADC and SCC by using this panel of immunomarkers. 19.6% of cases were categorized as poorly differentiated carcinoma.
Conclusion: Limited panel of IHC markers comprising of TTF1, p63 and CK7 can subtype a substantial percentage (80.4%) of poorly differentiated NSCLC.
Key words: Non-small cell lung carcinoma, Thyroid transcription factor-1(TTF-1), p63, Cytokeratin 7(CK7).

Introduction

Lung carcinoma being the 4th leading cause of death among all malignancies has a prevalence of 15% in Pakistan.[1] Discoveries of specific diagnostic tools and the emergence of targeted therapies (tyrosine kinase inhibitors) have highlighted the need to classify NSCLC into different subtypes, the more common of which are Adenocarcinoma (40%) and Squamous cell carcinoma (30%).[2]

As size of tissue obtained by transbronchial and endobronchial biopsies is small and there is a need to conserve tissue for further molecular studies, it becomes difficult to subtype those tumours which are poorly differentiated thus emphasizing the necessity for immunohistochemical staining. To increase the specificity and sensitivity, it is now recommended that a panel of antibodies should be used instead of a single one.[3]

When addressing this concern, International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) has derived a new approach to small biopsies with specific terms and criteria focused on the need for subtyping of NSCLC by immunohistochemistry (IHC). This is in contrast to the 2004 World Health Organization (WHO) classification of lung carcinoma which was based on the histological characteristics of resected tumours with little emphasis about diagnosis based on small biopsies.[4-6]

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Many studies have demonstrated a high sensitivity and specificity for p63 (95-96% & 86-90%), Thyroid Transcription Factor-1 [TTF1] (89-94% & 97-100%), and Cytokeratin 7 [CK 7] (80-92% & 76-91%) and are proven useful in differentiating the main subtypes of lung cancer which are SCC and ADC. [7, 8]

The rationale of this study was to evaluate the effectiveness of limited IHC panel in subtyping of poorly differentiated NSCLC in a resource limited setup and to help the clinicians and oncologists for giving precise treatment to the patients. [9]

The objective of this study was to see the expression of IHC markers TTF-1, CK7 and p63 in poorly differentiated NSCLC that could not be further classified as ADC or SCC in lung biopsy specimens on routine hematoxylin and eosin stained slides.

Methods

This study was conducted in Pathology department, Pakistan Institute of Medical Sciences (PIMS) Hospital, Shaheed Zulifqar Ali Bhutto Medical University Islamabad. The study was carried out from September 2015 to August 2016. It was a Cross-sectional study. A total of 51 cases of lung biopsies which were pan CK positive but were difficult to further classify as SCC or ADC on routine H&E staining were included by Non probability consecutive sampling. All the cases which were pan CK negative were excluded. After approval from hospital ethics committee and Advanced Studies and Research Board (AS&RB), Shaheed Zulfiqar Ali Bhutto Medical University, the lung biopsies were processed in the Department of Pathology, PIMS Hospital, Islamabad for histological and immunohistochemical evaluation. Patient's data was entered in the proforma. Results were interpreted in the light of the appropriate staining of positive controls. Only tumour cells stained in the appropriate nuclear or cytoplasmic location were scored. Immunoreactivity was scored semi quantitatively by recording the proportion and intensity of immunoreactive tumour cells.

- **Proportion of immunoreactivity in the study was scored as follow:**
  a = 0 (no positivity or only very occasional cell staining)
  b = 1+ (<10% of cells stained)
  c = 2+ (10%–50% of cells stained)
  d = 3+ (>50% of cells stained)
- **Intensity of immunoreactivity was scored as follows:**
  a = score 0, none (absent)
  b = score 1, weak (less than normal cells)
  c = score 2, moderate (same as normal cells)
  d = score 3, strong (stronger than normal cells)

For statistical analyses, only those cases with more than 10% (2+ or 3+) of tumour cells exhibiting definite (score 2 or score 3) staining were considered truly positive. Histologic (H) scores were derived by adding proportion scores (0, 1, 2, and 3) and intensity scores (0, 1, 2, and 3), yielding a number between 0 and 6. The positive cases were those having H score of ≥4.[10] Statistical analysis was performed by using SPSS Version 21. The numerical variables like age were analyzed as mean and standard deviation. The categorical variables like sex, IHC diagnosis and expression of IHC markers in ADC and SCC were calculated as proportions and percentages.

Results

Total number of cases were 51 (n=51). The age range of patients with NSCLC was between 28 to 94 years. The mean age of the patients was 59±14 years with a male to female ratio of 2:1. Out of the total 51 cases 26 males (51%) and 5 females (9.8%) were smokers. 46 cases (90.2%) presented with history of hemoptysis. 6 cases (11.8%) had history of biomass exposure and all were females.

Immunoreactivity of markers was evaluated on the basis of proportion of the cells stained and on intensity of the staining. 21 cases (41.2%) were positive for TTF-1 staining, 20 cases (39.2%) were positive for p63 staining and 26 cases (51%) were positive for CK7 staining. (Table 1) 41.2% of cases which showed positivity for TTF-1 were also positive for CK7. 5 cases (9.8%) were positive for CK 7 only and 5 cases (9.8%) were negative for all three markers. (Figure 1, 2, 3).

Table 1: Statistical Analysis of Immuno-reactivity (n=51)

Immuno-reactivity	H (Histological) Scoring	Frequency	%age
Interpretation of TTF 1 Immuno-reactivity	≥4 (positive)	21	41.2
	<4 (negative)	30	58.8
	Total	51	100
Interpretation of p63 Immuno-reactivity	≥4 (positive)	20	39.2
	<4 (negative)	31	60.8
	Total	51	100
Interpretation of CK 7 Immuno-reactivity	≥4 (positive)	26	51
	<4 (negative)	25	49
	Total	**51**	**100**

Final diagnosis based on the immunohistochemical findings was, that 20 cases (39.2%) which were positive for p63 only were that of lung squamous cell

carcinoma. 21 cases (41.2%), that were positive for both TTF-1 and CK7 were of lung adenocarcinoma.10 Cases (19.6%) which were only positive for CK7 or negative for all three markers (p63, TTF-1, CK7), were categorized as poorly differentiated carcinoma. (Table-2)

Table 2: Diagnosis based on H & E and IHC (n=51)

Diagnosis	Categories	Frequency	%age	
H & E Diagnosis	NSCLC Suggestive of SCC	35	68.6	
	NSCLC Suggestive of ADC	10	19.6	
	Poorly differentiated NSCLC	06	11.8	
IHC Diagnosis	SCC	20	39.2	**80.4**
	ADC	21	41.2	
	Poorly differentiated carcinoma	10	**19.6**	
Total No of Cases		51	100	

Table 3 shows the importance of IHC in diagnosing poorly differentiated NSCLC. Cases which had some features of SCC on H&E but were not fulfilling the criteria of SCC diagnosis like presence of intercellular bridges, keratin pearls and individual tumour cell keratinization were finally diagnosed as SCC or ADC after IHC staining. Same was the case with ADC.

Table 3: Utility of Immunohistochemistry in Sub-typing of Non-Small Cell Lung Carcinoma (n=51)

Impression on H & E Staining	Final Diagnosis after IHC	Frequency	%age
Squamous Cell Carcinoma	Squamous Cell Carcinoma	19	37.3
Squamous Cell Carcinoma	Adenocarcinoma	12	21.6
Adenocarcinoma	Adenocarcinoma	8	17.6
Squamous Cell Carcinoma	Poorly Differentiated NSCLC	4	7.8
Adenocarcinoma	Poorly Differentiated NSCLC	2	3.9
Poorly Differentiated NSCLC	Squamous Cell Carcinoma	1	2
Poorly Differentiated NSCLC	Adenocarcinoma	1	
Poorly Differentiated NSCLC	Poorly Differentiated NSCLC	4	7.8
Total		**51**	**100**

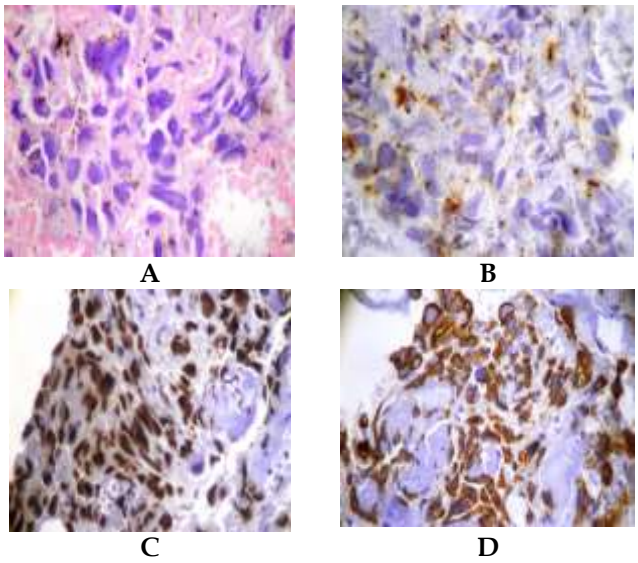

Figure 1: A: Suggestive of Squamous cell carcinoma on H&E X400, B: p63. Negative (0, score 0, Total score 0) X 400, C: TTF1 Positive (3+, score 3, Total score 6) X 400, D: CK7 Positive (3+, score 3, Total score 6) X400 Final Diagnosis on IHC: Adenocarcinoma

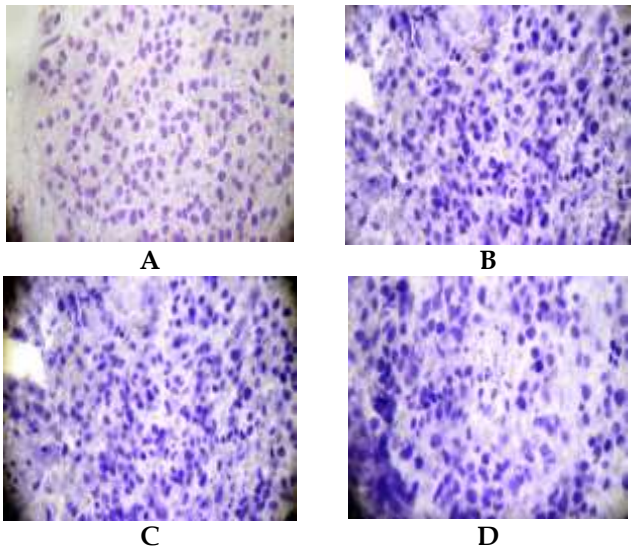

Figure-2: A: Suggestive of poorly differentiated NSCLC (H&E X 400), B: p63. Negative (0, score 0, Total score 0) X 400, C: TTF1 Negative (0, score 0, Total score 0) X 400, D: CK7 Negative (0, score 0, Total score 0) X 400
Final Diagnosis on IHC: Poorly differentiated carcinoma

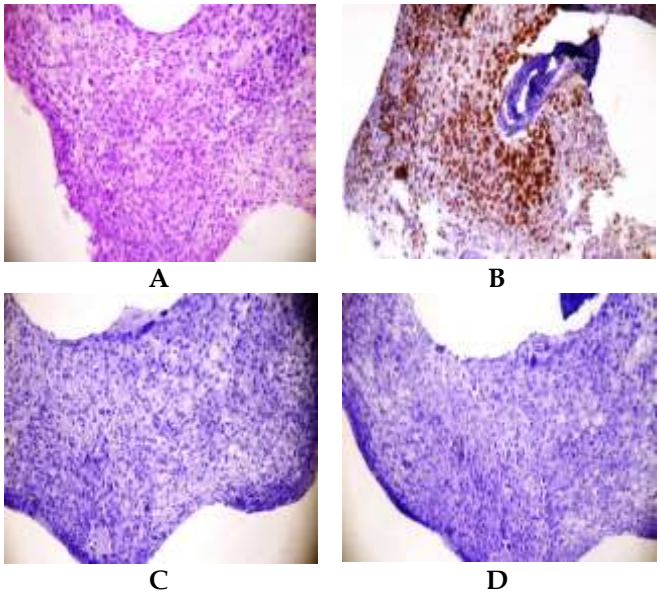

Figure-3: A: Suggestive of Squamous cell carcinoma H&E X100, B: p63. Positive (3+, score 3. Total score 6) X 100, C: TTF1 Negative (0, score 0, Total score 0) X 100, D: CK7 Negative (0, score 0, Total score 0) X 100 Final Diagnosis on IHC : Squamous cell carcinoma

Discussion

IHC is of great benefit for sub classifying lung carcinoma into different categories. As the biopsy material is scanty and there is a need that tissue should be preserved for molecular analysis, for example EGFR testing, the IHC panel should be carefully selected.

The age range of patients with NSCLC was between 28 to 94 years in the current study. The mean age was 59 years with a male to female ratio of 2:1. In a study conducted by Jafarian et al, the mean age of the patients was 60 years; ranged from 35 to 81 with a male to female ratio: 2.75: 1.[11] Moyer demonstrated that incidence of lung cancer increases with age and is most common in adults aged 55 years or older. [12]

26 male cases (51%) and 5 female cases (9.8%) out of the total 51 cases were smokers. Powell et al reported in a study that the proportion of heavy smokers was higher in men than in women (19% vs 15%). A higher proportion of women than men who developed lung cancer were recorded as never smokers (13% vs 8%).[13]

Lung carcinoma patients present with many specific and nonspecific symptoms like cough, hemoptysis, chest pain, fever, weight loss and shortness of breath etc. In the current study 46 cases (90.2%) out of the total 51 cases presented with history of hemoptysis and all patients had history of cough. Walter et al in a study of 153 cases demonstrated that 33 (21.6%) of the patients presented with hemoptysis as first symptom and 86% patients had history of cough. [14]

In the current study 6 out of 17 females (11.8%) presented with a history of biomass exposure. Biomass exposure is mostly associated with chronic obstructive pulmonary disease but association with lung carcinoma is also demonstrated. In a study by Arrieta et al on 914 lung cancer patients, 35% had a background of wood smoke exposure. [15]

Ezzat demonstrated that 75% of NSCLCs need only routine H&E staining to be diagnosed as ADC or SCC in biopsy specimens.[16] Problem arises in those cases which show poorly differentiated morphology. Current study was designed to see the importance of the limited panel of IHC markers constituting of TTF-1, p63 and CK7 in classifying poorly differentiated NSCLC.

Several studies have described the use of same immunomarkers for sub-typing NSCLC which were used in the present study. Shankar et al demonstrated that a panel of IHC markers, TTF-1 and p-63, help in sub-typing the poorly differentiated NSCLC.[17] Similarly Noh et al studied the use of TTF-1, p-63 and CK7 for classifying NSCLC.[18]

In the current study 21 cases (41.2%) showed H score of ≥4 and these were considered positive for TTF-1 staining. These 21 cases were finally diagnosed as lung ADC because all of them also stained positive for CK7. Gurda et al in their study showed that TTF-1 was positive in 60/71 ADC. (Sen 84.5%, sp 96.4%). [19] Kim et al showed in their study that TTF-1 staining was positive in 57 (70%) of 81 ADC specimens . [20] Xu et al conducted a study about IHC profile of NSCLC and staining for TTF-1 was present in 105 of 111 Adenocarcinoma.[8]

20 cases (39.2%) showed H score of ≥4 and these were considered positive for p63 staining. Gurda et al in their study showed that p63 was positive in 22/24 SCC. (Sen 91.7%, sp 78.3%). [19] Kim et al showed p63 staining was positive in 43 (90%) of 58 SCC specimens. [20] Xu et al showed that p63 was positive in 96 of 99 SCC. [8] When considering these previous studies, it can be well established that p63 is a sensitive and specific marker for lung SCC and 20 cases in our study which had p63 H score ≥4 are that of SCC.

26 cases (51%) showed H score of ≥4 and these were considered positive for CK7 staining. Gurda et al in their study showed that CK 7 was positive in 45/48 ADC. (Sen 93.8%, sp 50%). [19] Xu et al (n=111 SCC and 99 ADC) showed that CK7 was positive in 89 of 111 ADC. CK7 had a lower sensitivity (80.18%) and specificity (91.92%) than TTF-1 (sensitivity 94.59%,

specificity 100%). They showed that the sensitivity (98%) and specificity (100%) of the combination ofCK7/TTF-1 were almost the same as those of TTF-1 alone. [8] Ma et al showed in their study that the sensitivity and specificity of CK7 in detecting lung ADC and distinguishing it from lung SCC were 94.6 and 76.0 % respectively. [21] This means that CK7 alone does not always show that a tumour can be of lung origin. It can be metastatic.

According to Mukhopadhyay, CK7 for ADC is highly sensitive but too non-specific to be useful. It is positive in almost all poorly differentiated ADC but it also stains 60% of poorly differentiated SCC. Therefore, positivity for CK7 is of no utility for NSCLC sub classification. It is significant to put emphasis on that even though CK7 is unsupportive in sub classification of poorly differentiated NSCLC, this does not weaken its utility in other situations. For example, if a tumour in a small lung biopsy is assumed to be metastatic based on morphology, clinical history or radiologcal findings, CK7 can be an important marker as part of a panel to decide a primary site.[22]

Montezuma et al proposed panel (CK7, CK20, TTF-1, and p63) allowed accurate histological sub classification of 87% of NSCLC cases, which is close to the lower range of the predicted 10–30% of NSCLC-NOS cases diagnosed in small biopsies/cytology samples. [7] Same was the case in the present study. 80.4% of NSCLC were classified as ADC and SCC because they had the expression of TTF-1 and CK 7 or p63. 19.6% of the cases in the current study could not be further classified because of the CK7 positivity only or negativity of all three markers.

Zachara et al in their study concluded that IHC staining allows accurate subclassification of poorly differentiated NSCLCs on small lung biopsies in most cases, but 18% of the tumours still remain undiagnosed. [23] In the current study this percentage was 19.6%. On small biopsies, such tumours should be diagnosed as 'poorly differentiated non-small cell lung carcinoma', or 'non-small cell lung carcinoma, not otherwise specified' (NSCLC, NOS). In this study they were labelled as poorly differentiated carcinoma as they can be of lung origin or metastatic and an extended panel of IHC markers should be used to subtype them.

Napsin A has emerged as a more specific marker for lung ADC when compared to TTF-1. Gurda et al demonstrated that taken together, TTF-1 had a better sensitivity, and Napsin A had a better specificity for the primary lung ADCs. Whereas, CK7 showed a suboptimal specificity for lung ADCs.[19]

Current study was also of great help in those cases which had some features of SCC on morphology but were re-diagnosed as ADC after IHC staining or vice versa, which concludes that it is mandatory to apply ancillary studies on every suspicious case so that proper diagnosis is made for further treatment options. Osmani et al demonstrated that some lung ADC with solid growth pattern can be confused with non-keratinizing SCC on small biopsy specimens. In certain small biopsy specimens, the subclassification of the tumour, in addition to the morphologic evaluation and immunohistochemical characteristics of the tumour, may be still difficult. For these reasons, the guidelines emphasize the critical role of IHC markers in the accurate subclassification of lung tumours in addition to morphological evaluation, particularly in small biopsy specimens.[24, 25]

Current study encompasses only those cases in which history and morphological findings were consistent with tumour of pulmonary origin and suggestive of NSCLC on H&E. IHC panel for NSCLC should be planned after a careful judgment of morphological picture on H&E and after excluding small cell carcinoma and metastatic malignant tumours based on clinical history and radiological findings.

A limitation of this study was its small sample size, which can result in bias. Subsequent surgical resection specimens of the same patients were not available for the verification of the Immunohistochemical findings. Limited possible immunohistochemical workup was done because tissue should be preserved for further EGFR genetic testing.

As the sample size was small in the current study, similar studies on large sample size are recommended to rule out the bias.

Further work should be done regarding the molecular testing of EGFR in cases of lung ADC because of the new targeted therapies for different types of Non-Small Cell Lung Carcinoma.

Large scale studies to provide a data about incidence and prevalence of lung carcinoma in Pakistani population are recommended because up till now no sufficient data set is available.

Conclusion

A substantial percentage of poorly differentiated NSCLC can be subclassified as SCC and ADC on small lung biopsies using a panel comprising of TTF1, p63 and CK7. According to current study up to 80.4% of NSCLC were sub-typed by using this limited panel of immunomarkers. Positivity for CK7 is of no utility

alone but it can be used as a supportive marker of TTF-1 in Lung ADC. It was also observed that cases which had some features of SCC on morphology, turned out to be ADC after IHC staining, which concludes that it is mandatory to apply ancillary studies (IHC) on every suspicious case so that proper diagnosis be made because of the availability of targeted therapies for specific tumour subtypes.

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AUTHOR CONTRIBUTION:

Rabiya Fawad: Conception, study design, study conduction, analysis, interpretation, manuscript writing and review.
Maryam Qaiser: Study conduction, analysis, writing and **facilitated in materials.**
Ashok Kumar Tanwani: Conception, study design, analysis and critical review
Ahmareen Khalid Sheikh: Study conduction, interpretation, facilitated in materials
Saira Javed: Interpretation and critical review