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A comparative analysis of P40 and P63 immunomarkers for differentiation of squamous cell carcinoma and adenocarcinoma, lung

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Abstract
Distinguishing between lung adenocarcinoma and squamous cell carcinoma is becoming increasingly important, as discovery of new advent of targeted therapies, further subtyping of NSCLCs has profound therapeutic implications. Until recently a panel of TTF-1/p63 immunostains is the current recommendation for differentiation of ADC from SqCC in small biopsies or cytological specimens. However, studies using antibodies against p63 have demonstrated false-positive results with positivity in some ADC. P40, a relatively new antibody that targets one p63 isoform - ΔNp63, has been shown a promising marker in identifying SqCC with high sensitivity and specificity compare to P63. In this study, we compared the standard P63 antibody with P40 immunoreactivity in the 160 cases of primary lung carcinoma which included lung ADCs (n=86) and SqCCs (n=74). The p63 was positive in 98.65% of squamous cell carcinomas and 9.23% of adenocarcinomas (sensitivity 98.65%, specificity 93.02%). In contrast, although p40 was also positive in 100% of squamous cell carcinomas, only 1.54% of adenocarcinomas had p40 labeling (sensitivity 100%, specificity 98.83%). Rare adenocarcinomas with p40 labeling had reactivity in no more than 5% of tumor cells, whereas p63-positive cells in adenocarcinomas was >50%. In summary, p40 appears to be a more reliable marker for squamous cell carcinoma as equivalent to p63 in sensitivity, but it is markedly superior to p63 in specificity, which eliminates a potential pitfall of misinterpreting a p63-positive adenocarcinoma as squamous cell carcinoma. In this study, our findings strongly support the routine use of p40 in place of p63 for the diagnosis of pulmonary squamous cell carcinoma.

Keywords: squamous cell carcinoma, adenocarcinoma, DNp63; p40; p63

Introduction
Lung cancer is the leading cause of cancer related death worldwide and accounts for 28% of all cancer mortality [1] and around 1.8 million new cases were diagnosed in 2012. [2] In India, lung cancer constitutes 6.9 percent of all new cancer cases and 9.3 percent of all cancer related deaths. [3]

Several histotypes of lung cancer exist, among them majority are non-small cell carcinomas (NSCLCs) including adenocarcinoma (ADC) and squamous cell carcinoma (SqCC). In light of discovery of molecular alterations associated with lung ADCs and advent of targeted therapies, further subtyping of NSCLCs has profound therapeutic implications. [4, 5, 6] In most cases, the distinction of ADC and SqCC is readily achieved based on standard morphologic criteria, with keratinization and intercellular bridges representing hallmarks of SqCC and glandular architecture (in the form of acini, papillae, micropapillae, or cytoplasmic mucin) representing the hallmarks of ADC. [7] Differentiation of lung ADCs from SqCCs may sometimes be difficult to achieve based on histomorphology alone, especially it can be challenging in poorly differentiated tumors. This issue is particularly amplified in small specimens (small biopsies and cytology) where focal evidence of morphologic differentiation may not be represented as a result of scant cellularity, crush artifact, or cell dispersal and may require a panel of immunohistochemistry stains.

Until recently, a non-committal diagnosis of NSCLC - NOS was widely advocated as a general approach to small specimens because of the inability of morphology to distinguish some poorly differentiated tumors combined with the lack of clinical significance. Recently, as a result of the 'histology-based' molecular and therapeutic advances, there has been a

major paradigm shift in the approach to pathologic diagnosis of NSCLC with a new emphasis now placed on specific and accurate NSCLC sub typing in small specimens. [7, 8] This new approach to NSCLC diagnosis is advocated in the recent IASLC/ATS/ERS lung adenocarcinoma classification. [9] In the past 30 years, immunohistochemistry (IHC) has been able to show the proteins expressed by different tumors and has given refinement to classification [10, 11]

In order to meet above challenge, immunohistochemistry (IHC) has been shown to be a valuable adjunct to H&E staining, particularly for poorly differentiated/ undifferentiated tumors. [12] To make more effective use of limited tissue, multiplex IHC approaches have been developed, wherein two or more antibodies directed against morphologically distinct antigens are added to the same tissue sample. [13, 14] Each antibody can be detected using a different color chromogen.

P63 has emerged as the 'front runner' of the squamous markers. A panel of TTF-1/p63 immunostains is the current recommendation for differentiation of ADC from SqCC in small biopsies or cytological specimens. However, studies using antibodies against p63 have demonstrated false-positive results with positivity in some ADC. Several studies have shown that p63 has an extremely high sensitivity (approaching 100%) for SqCC. [8] However, the main limitation of p63 is low specificity due to its unexpected reactivity in 16–65% of lung ADC. [8, 15] Another important limitation of p63 as a 'squamous marker' is its unexpected expression in several other tumor types, particularly lymphomas, [8] where reactivity has been reported in up to half of the cases. Keeping in view the low specificity of P63 at present "A diffusely positive P63 with absence of TTF-1", is accepted as an indicator of SqCC differentiation. Therefore P63 is dependent upon TTF-1 for diagnosis of SqCC.

The anti-p40 antibody, a relatively new immunomarker, has been reported in several studies for the distinction of lung SqCC and ADC, suggesting that unlike p63 antibody, p40 antibody is highly squamous-specific. [8] And thus the anti-p40 antibody has been recommended instead of the anti-p63 antibody for the diagnosis of pulmonary SqCC.

The present study aims at evaluation of P40, a relatively new immunomarker, and compares its sensitivity and specificity with p63, a commonly used immunomarker in sub typing of NSCLC into SqCC and ADC.

Materials and Methods

A total of 160 primary lung carcinoma cases included over a period of two years with unequivocal morphological diagnosis irrespective of age, gender and nature of biopsy material (endoscopic biopsy/ needle core biopsy / resected specimen). Cases diagnosed as Small cell carcinoma of lung, as metastatic lung cancers, poorly differentiated NSCLC-NOS and with inadequate material for IHC study were excluded from present study.

Among 160 cases, 86 adenocarcinomas and 74 squamous cell carcinomas were diagnosed by Hematoxylin (Meyer's) and Eosin stain. The grading were rendered on the basis of broder's grading system and classified into well and moderately differentiated SqCC and ADC. Among ADC, 21 cases were well and 65 cases were moderately differentiated where as in SqCC, 16 cases were well and 58 cases were moderately differentiated SqCC. IHC Analysis were done by using antigen retrieval method: BIO GENEX-EZ-Retriever system V.2 (temperature controlled microwaving). Percentage of SqCC and ADC showing positivity for P40 and P63 immunomarker and Sensitivity and specificity was calculated.

Immunohistochemistry with p40 antibody was performed at in department of santokbha durlabhji memorial hospital cum research centre, Jaipur. Antigen retrieval was performed with CC1 buffer (Cell Conditioning 1; citrate buffer pH 6.0, Ventana Medical Systems). Immunohistochemistry for p63 (TP63; 4A4, Dako, 1:700 dilution) was performed. P63 (4A4) recognizes an epitope shared by TAp63 and DNp63 isoforms, whereas p40 recognizes an epitope which is unique to DNp63. For all markers, both extent (% cells) and intensity (1+, 2+, and 3+) of immunoreactivity were recorded. Only nuclear immunoreactivity was accepted. The P-values 'p' <0.05 was taken as significant. MedCalc Software 16.4 version was used for all statistical calculations.

In addition, 10 cases of poorly differentiated non-small cell (large cell) carcinomas where the tumor predominantly showed solid growth with no apparent squamous or glandular differentiation, and the diagnosis of poorly differentiated (large cell) carcinoma was made and final diagnosis was suggested purely on the basis of immunohistochemistry findings: that is, TTF-1, p63 and p40 expression.

Results

NSCLC constituted 83.84% (337/402) and SCLC 14.42% (58/402) of all lung carcinoma in the present study. Majority of cases 44.03% (177/402) of NSCLC, in the present study, were unclassified and grouped under NSCLC- NOS. 160 cases with unequivocal morphologic diagnosis of "SqCC and ADC" were included in the study for further evaluation. SqCC was diagnosed in 46.25% (74/160) of cases, graded as WDSqCC (21.62%) and MDSqCC (78.38%). ADC was diagnosed in 53.75% (86/160) cases, graded as WDADC (24.41%) and MDADC in (75.59%) cases (table-2). P63 positivity was observed in 73/74 cases of SqCC – the sensitivity of P63 being 98.65%. P63 positivity was observed irrespective of grade of SqCC, as in WDSqCC (93.75%), 100% in MDSqCC and 100% in PDSqCC (table-3) (figure-1). All P63 positive SqCC showed homogenously diffuse positivity (>50% tumor cells) with strong intensity (2+ and 3+) in all cases (table-4). The reactivity for p63 was consistently strong and diffuse in all positive cases of SqCC. The single P63 negative SqCC case (1.35%) was of well differentiated grade.

P63 was negative in 80/86 cases of ADC – the Specificity of P63 for diagnose of lung SqCC being 93.02%. P63 was positive in 6/86 (9.23%) ADC and this positivity was irrespective of grade, expressed in >50% tumor cells—the extent typical of SqCC along with strong intensity 2+ in all cases (table-4) figure-2 & 3). The p63 reactivity in ADC was undistinguishable from the diffuse reactivity for p63 in SqCC.

P40 positivity was observed in 74/74 cases of SqCC – the Sensitivity of P40 being 100%. P40 positivity was observed irrespective of grade of SqCC, as 100% in WDSqCC, 100% in MDSqCC and 100% in PDSqCC (table-5) (figure-1). All P40 positive SqCC expressed homogenously diffuse

positivity (>50% tumor cells) with strong intensity (2+ and 3+) in all cases (table-6). The reactivity was consistently strong and diffuse in all P40 positive SqCC cases. P40 was negative in 85/86 cases of ADC– the Specificity of P40 for diagnose of lung SqCC 98.83%. The single (1.54%) P40 positive cases of ADC was of moderately differentiated grade and showed minimal reactivity (<5% of tumor cells) with minimal staining intensity (table-6) (figure-2 & 3). This minimal p40 reactivity in rare ADC is readily distinguishable from the diffuse reactivity for p40 in SqCC, hence can't be confused with SqCC.

Sensitivity of p40 was almost equivalent to sensitivity of p63 for SqCC (100% vs 98.65%) (table-8). Majority of SqCC were positive for both markers, and reactivity for both markers was consistently diffuse and strong. The extent and intensity of p40 reactivity in SqCC were indistinguishable from that of p63.

P40 was markedly superior to p63 in specificity (98.98% vs 93.02%) (table-8). P40 positive ADC in 1.54 % (1/86) of case showed focal positivity (<5% extent) and weak intensity.

PPV of P40 for diagnosis of SqCC was 98.66% and for P63 was 90.40%. NPV of P40 for diagnosis of SqCC in present study was 100% and for P63 was 98.76% (table-8).

10 cases of NSCLC- NOS were randomly selected in present study. P63 and TTF-1 antibodies were used for sub typing of NSCLC-NOS. The achieved results were reviewed for P40 expression: Five cases were positive for P63 and negative for TTF1 antibody. These were diagnosed as SqCC. Four cases were negative for P63 and positive for TTF1 antibody. These were diagnosed as ADC.

One case was diffuse positive for both P63 and TTF1 antibody. In view of strong TTF1 positivity, the P63 positivity could not be interpreted and equivocal diagnosis of NSCLC-NOS remained as such. Application of P40 expressed no reactivity, knowing the high specificity of P40, the case was confirmed as ADC. This case was P63 positive ADC and could have been misdiagnosed as SqCC, if TTF1 was not applied. This stresses the fact that P63 is dependent on TTF1 for accurate diagnosis and cannot be used alone as a single antibody. P40 on the other hand is not dependent on TTF1 and can be used as a single marker – This requires further validation on a larger study.

Table 1: Distribution of lung carcinoma according to age (n = 160) (Age wise distribution of the cases)

Age	No.	%
≤40	5	3.13
>40	155	96.87
Total	160	100.00

Out of 160 cases of lung carcinoma, 155 (96.87%) cases were diagnosed in age more than 40 years.

Table 2: Grading of lung carcinomas (Broder's Grading) (n=160) (Grading of lung carcinoma)

Morphological Diagnosis	Grade/differentiation	No.	%
SQCC (74)	Well differentiated SQCC	16	21.62
	Moderately differentiated SQCC	58	78.38
ADC (86)	Well differentiated ADC	21	24.41
	Moderately differentiated ADC	65	75.59
	Total	160	100.00

- Out of total 74 cases of SqCC, 16 (21.62%) cases were well differentiated SqCC and 58 (78.38%) cases were moderately differentiated SqCC.
- Out of total 86 cases of ADC, 21 (24.41%) cases were well differentiated ADC and 65 (75.59%) cases were moderately differentiated ADC.

Table 3: Correlation of P63 Immunomarker expression with morphologic type of lung carcinoma (n =160) (P63 expression in subtype of carcinoma)

Final Diagnosis	Total	P63 Negative		P63 Positive	
		No.	%	No.	%
MDSqCC	58	0	0.00	58	100.00
WDSqCC	16	1	6.25	15	93.75
MDLADC	65	59	90.77	6	9.23
WDLADC	21	21	100.00	0	0.00

- All 58 (100%) cases of moderately differentiated SqCC showed P63 nuclear positivity.
- Out of 16 cases of well differentiated SqCC, 15 (93.75%) cases showed p63 nuclear positivity but one (6.25%) case was P63 negative.
- Out of 65 moderately differentiated ADC, 59 (90.77%) cases were P63 negative but 6 (9.23%) cases showed P63 positivity.
- Whereas all 21 cases of well differentiated ADC were P63 negative.

Table 4: Distribution in 160 Cases of expression of P63 Immunomarker in lung carcinoma depiciting intensity of Staining. (n=160) (P63 intensity of staining)

Final Diagnosis	Total	P63 Intensity 1+		P63 Intensity 2 +		P63 Intensity 3+	
		No.	%	No.	%	No.	%
MDSqCC	58	0	0.00	20	34.48	38	65.52
WDSqCC	15	0	0.00	5	33.33	10	66.67
MDLADC	6	6	100.00	0	0.00	0	0.00
WDLADC	0	0	0.00	0	0.00	0	0.00

- Out of 58 cases of P63 positive moderately differentiated SqCC, 38 (65.52%) cases showed 3+intensity and 20 (34.48%) cases showed 2+ intensity.

- Out of 15 cases of P63 positive well differentiated SqCC, 10 (66.67%) cases showed 3+ intensity and 5 (33.33%) cases showed 2+ intensity.
- Whereas P63 positive 6 cases of moderately differentiated ADC showed 1+ intensity. All well differentiated cases of ADC were P63 negative

Table 5: Correlation of P40 Immunomarker expression with morphologic type of lung carcinoma. (n=160) (P40 immunomarker expression)

Final Diagnosis	Total	P40 Negative		P40 Positive	
		No.	%	No.	%
MDSqCC	58	0	0.00	58	100.00
WDSqCC	16	0	0.00	16	100.00
MDLADC	65	64	98.46	1	1.54
WDLADC	21	21	100.00	0	0.00

- All cases of SqCC 74 (100%) showed nuclear positivity for P40 antibody which includes 16 (21.62%) cases of well differentiated SqCC and 58 (78.38%) cases of moderately differentiated SqCC
- 85 (98.46%) cases out of 86 Cases of ADC showed P40 immunomaker negative except one (1.54%) case of moderately differentiated ADC which was P40 positive.

Table 6: Expression of P40 Immunomarker in lung carcinoma depiciting intensity of Staining (n=160) (P40 intensity of staining)

Final Diagnosis	Total	P40 Intensity 1+		P40 Intensity 2+		P 40 Intensity 3+	
		No.	%	No.	%	No.	%
MDSqCC	58	0	0.00	10	17.24	48	82.76
WDSqCC	16	0	0.00	4	25.00	12	75.00
MDLADC	1	1	100.00	0	0.00	0	0.00
WDLADC	0	0	0.00	0	0.00	0	0.00

- Among the 58 cases of moderately differentiated SqCC which were positive for P40, 10 (17.25%) cases showed 2+ intensity and and rest 48 (82.76%) cases were show 3+ intensity.
- Out of 16 cases of well differentiated SqCC, 4 (25%) cases showed 2+ intensity and 12 (75%) cases showed 3+ intensity.
- Only one case of MD ADC was P40 positive showed extremely low intensity (1+).

Table 7: Concordance between morphological diagnosis and P40 and P63 IHC diagnosis (morphologic diagnosis and IHC diagnosis)

Final Diagnosis	Total Positives	P40		P63	
		No.	%	No.	%
MDSqCC	58	58	100.00	58	100.00
WDSqCC	16	16	100.00	15	93.75
MDLADC	65	1	1.54	6	9.23
WDLADC	21	0	0.00	0	0.00

- All 58 (100%) cases of moderately differentiated SqCC were positive for P40 and P63 antibody.
- All 16 (100%) cases of well differentiated SqCC were positive for P40 antibody and out of 16 cases of WD SqCC, 15 (93.75%) cases were P63 positive.
- Out of 65 cases of moderately differentiated ADC, 1(1.54%) case was positive for P40 antibody whereas 6 (9.23%) cases were positive for p63 antibody.
- All 21 (100%) cases of well differentiated ADC were negative for P40 and P63 expression.

Table 8: Comparative analysis of P40 and P63 Antibodies in Sub classification of NSCLC (Final Diagnosis)

	P40		P63		'p' Value*
	No.	%	No.	%	
Sensitivity	74/74	100.00	73/74	98.65	0.993
Specificity	85/86	98.83	80/86	93.02	0.123
PPV	74/75	98.66	73/79	90.40	0.060
NPV	85/85	100.00	80/81	98.76	0.984
Accuracy	159/160	99.37	153/160	95.62	0.074

*'Z' test to Compare Two Proportions

- In this study, the sensitivity of P63 and P40 for SqCC was 98.65% and 100% respectively.
- Specificity of P63 and P40 was 93.02% and 98.83% respectively.
- Positive predictive value of P63 and P40 was 90.40% and 98.66% repectively.
- Negative predictive value for P63 and P40 was 98.76% and 100% respectively.
- So the accuracy calculated for P63 and P40 is 95.62 % and 99.37% respectively.

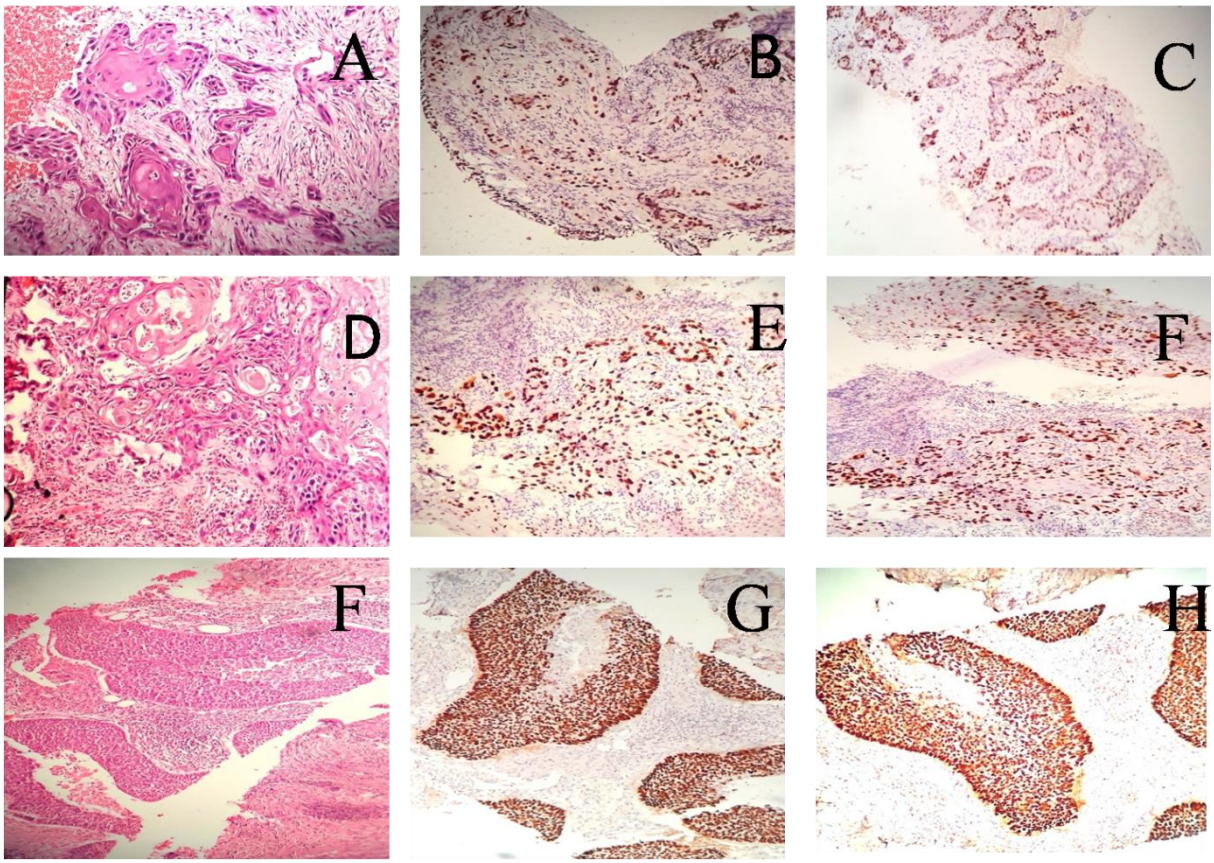

Fig 1: Well, moderately and poorly differentiated squamous cell carcinoma with IHC expression. A.& D Photomicrograph showing Well & Moderately differentiated squamous cell carcinoma consists of anastomosing cords of neoplastic cells with areas of keratinization with p40 and p63 expression. A & D (H&E, X20). B & C. P40 & P63 show intense positive staining that highlights the tumor cells in well differentiated sqcc. (P40 & p63, x20) E & F. P40 & P63 show intense positive staining that highlights the tumor cells in moderately sqcc.(P40 & p63, x20). F. Photomicrograph of poorly differentiated squamous cell carcinoma showing highly mitoticaly active cells infiltrating the stroma and moderate inflammatory response. G. Tumor cells showing diffuse and intense nuclear immunoreactivity for P40 & P63. (P40 & P63, x20)

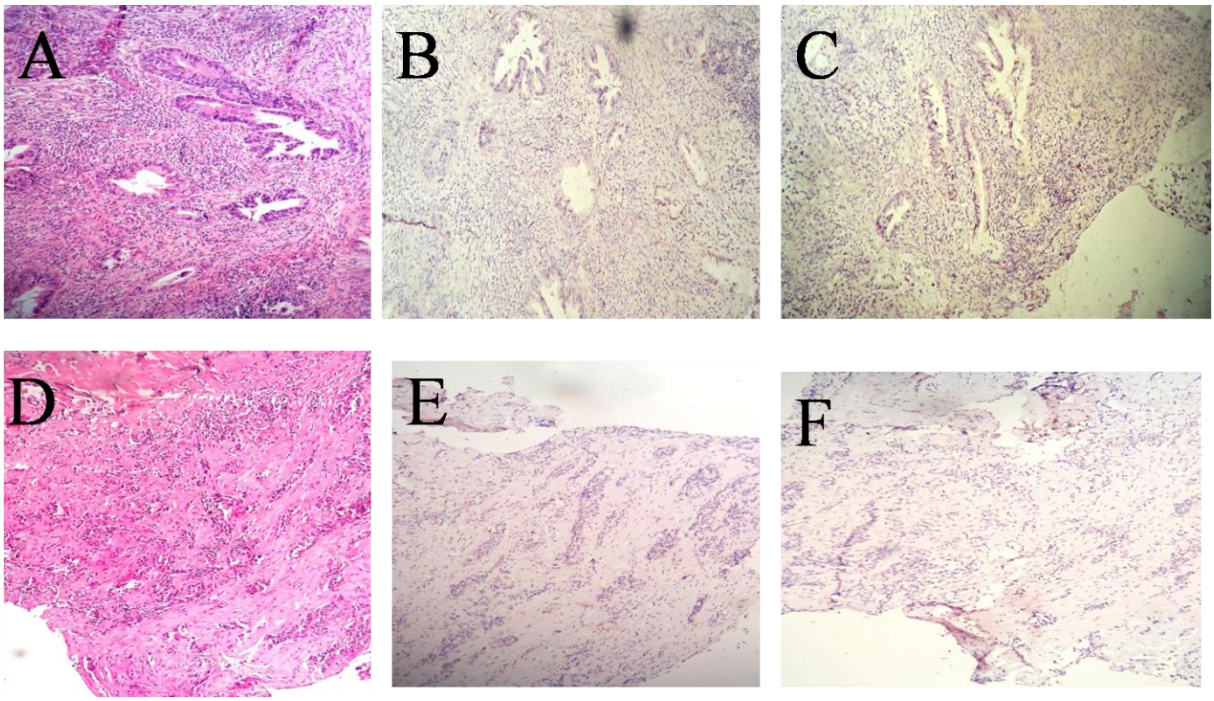

Fig 2: Moderately and poorly differentiated Adenocarcinoma with IHC expression. A -Photomicrograph showing moderately differentiated ADC comprising of glands lined by uniform cells with basal nuclei and abundant intracytoplasmic mucin (H& E x20). B & C -Tumor cells are negative for P40 and P63 immunomarker (P40 and P63, X20) D- Photomicrograph of bronchial biopsy showing poorly differentiated tumor cells arranged in solid sheets. (H&E x20). E & F - B.tumor cells showing no reactivity for P40 and P63 (P40 and P63, x20)

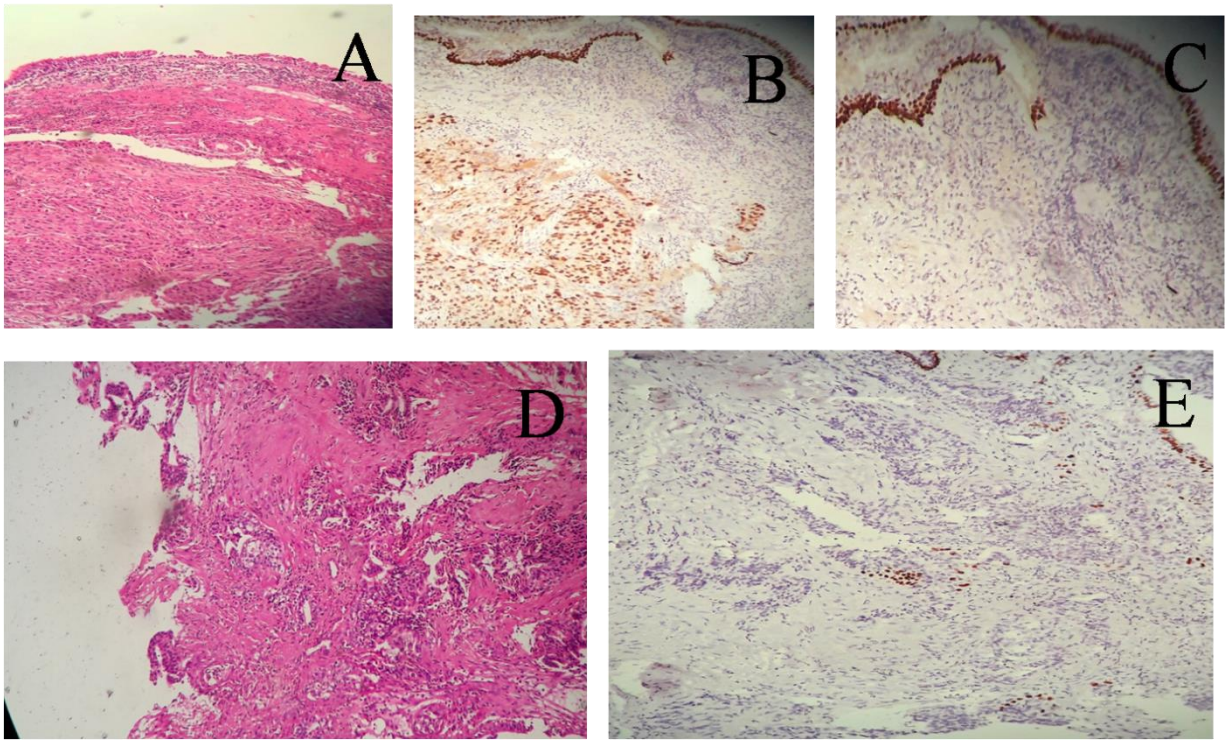

Fig 3: Moderately differentiated ADC with unusual expression of IHC markers. A- Photomicrograph of bronchial biopsy showing poorly differentiated tumor cells arranged in solid sheets.(H&E x20) B -Rare ADC case showing diffuse P63 positivity for tumor cells (p63, x20) & C- tumor cells shows no reactivity for P40 (P40, x40) D. Photomicrograph of moderately differentiated ADC shows Tumor cells infiltrating the bronchial muscosa singly, in small nests and glands.(H&E X20) E-Uncommon lung ADC showing focal nuclear staining with P40 immunomarker. (H & E X20)

Discussion

Our study revealed that p40 was the best marker for differentiating pulmonary SQC from non-SQC. The immunoreactivity of p40 in non-SQCs was focal and faint compared with diffuse strong reactivity in SQC. The sensitivity of p40 for diagnosing pulmonary SQC was found to be 100% in the present study (74/74; 100%) similar in previous reports (Bishop *et al* [8] and Nonaka D. [15]). The sensitivity of widely used p63 (TAp63 isoforms) for diagnosing pulmonary SQC were 98.65% (73/74) whereas its 100% in previous report of Bishop *et al* [8] and Nonaka D. [15]. The single P63 negative SqCC case 1.35% was of well differentiated grade. This negativity cannot be explained, we are putting our observation forward that the carcinoma showed extensive keratinisation with keratin flakes and mature keratinocytes which requires further validation.

The reported specificity of p40 in the present study was 98.83% and ranged from 88% to 100% in previous studies (Bishop *et al* [8] and Nonaka D. [15] and Pelosi G [16]). The single P40 positive cases of ADC 1.54% was of moderately differentiated grade and showed minimal reactivity (<5% of tumor cells) with minimal staining intensity. This minimal p40 reactivity in rare ADC is readily distinguishable from the diffuse reactivity for p40 in SqCC, hence can't be confused with SqCC. The reported specificity of p63 in the present study was 93.02%. P63 was positive in 9.23% (6/86) ADC and this positivity was irrespective of grade, expressed in >50% tumor cells the extent typical of SqCC along with strong intensity 2+ in all cases. The p63 reactivity in ADC was undistinguishable from the diffuse reactivity for p63 in SqCC.

Sensitivity of p40 was almost equivalent to sensitivity of p63 for SqCC (100% vs. 98.65%) Majority of SqCC were positive for both markers, and reactivity for both markers was consistently diffuse and strong. The extent and intensity of p40 reactivity in SqCC were indistinguishable from that of p63. Whereas, P40 was markedly superior to p63 in specificity (98.98% vs 93.02%). P40 positive ADC in 1.54% of case showed focal positivity (<5% extent) and weak intensity. These few positive cells could be entrapped basal cells which were detected by DNp63 isoforms of P40 – if, the low positive can be disregarded then specificity of P40 is virtually 100%.

In the pilot study of 10 cases of NSCLC-NOS - It was observed that P63 is dependent on TTF1 for accurate subtyping of NSCLC-NOS into SqCC and ADC. It is of paramount importance to use P63 always in combination with TTF1 (glandular marker) to prevent misdiagnosis of P63 positive ADC as SqCC. However, P40 was found not to be dependent on TTF1 and can be used alone as a single marker for diagnosis of SqCC – This require further validation in larger study.

Conclusion

In conclusion, we find that p40 is equivalent to p63 in sensitivity for pulmonary squamous cell carcinoma, but it has a markedly superior over p63 in view of specificity. In rare cases in which p40 labeling is seen in adenocarcinoma, it is very focal, limited to isolated tumor cells, which is readily distinguishable from the diffuse reactivity in squamous cell carcinomas. Diffuse labeling with p40 supports diagnosis of SqCC (PPV 98.66%) and absence of reactivity is a strongly against diagnosis of SqCC (NPV 100%). We suggest that a strong consideration should be given for a routine use of p40 in place of p63 as a marker of pulmonary squamous cell carcinoma.

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