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p40 (ΔNp63) is superior to p63 for the diagnosis of pulmonary squamous cell carcinoma

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Immunohistochemistry has recently emerged as a powerful ancillary tool for differentiating lung adenocarcinoma and squamous cell carcinoma—a distinction with important therapeutic implications. Although the most frequently recommended squamous marker p63 is extremely sensitive, it suffers from low specificity due to its reactivity in a substantial proportion of lung adenocarcinomas and other tumor types, particularly lymphomas. p40 is a relatively unknown antibody that recognizes ΔNp63—a p63 isoform suggested to be highly specific for squamous/basal cells. Here we compared the standard p63 antibody (4A4) and p40 in a series of 470 tumors from the archives of Memorial Sloan–Kettering Cancer Center and The Johns Hopkins Hospital, which included lung squamous cell carcinomas ($n=81$), adenocarcinomas ($n=237$), and large cell lymphomas ($n=152$). The p63 was positive in 100% of squamous cell carcinomas, 31% of adenocarcinomas, and 54% of large cell lymphomas (sensitivity 100%, specificity 60%). In contrast, although p40 was also positive in 100% of squamous cell carcinomas, only 3% of adenocarcinomas, and none of large cell lymphomas had p40 labeling (sensitivity 100%, specificity 98%). The mean percentage of p63 *versus* p40-immunoreactive cells in squamous cell carcinomas was equivalent (97 *vs* 96%, respectively, $P=0.73$). Rare adenocarcinomas with p40 labeling had reactivity in no more than 5% of tumor cells, whereas the mean (range) of p63-positive cells in adenocarcinomas and lymphomas was 26% (1–90%) and 48% (2–100%), respectively. In summary, p40 is equivalent to p63 in sensitivity for squamous cell carcinoma, but it is markedly superior to p63 in specificity, which eliminates a potential pitfall of misinterpreting a p63-positive adenocarcinoma or unsuspected lymphoma as squamous cell carcinoma. These findings strongly support the routine use of p40 in place of p63 for the diagnosis of pulmonary squamous cell carcinoma.
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Until recently, the only clinically significant histological distinction for lung cancers was between small cell carcinoma and non-small cell carcinoma. With the introduction of novel targeted therapies that are used differentially in lung adenocarcinoma and squamous cell carcinoma—the two most common types of non-small cell lung carcinomas—the precise distinction of these tumor types has become imperative. For example, the small molecule EGFR inhibitors erlotinib and gefitinib are only indicated in non-small cell lung carcinomas with *EGFR* mutations, which occur almost exclusively in adenocarcinoma.[1] Similarly, the recently identified *EML4-ALK* rearrangement, which predicts susceptibility to the targeted agent crizotinib, also occurs only in adenocarcinoma.[2] In addition, the angiogenesis inhibitor bevacizumab and the folate antimetabolite pemetrexed are excluded from use in patients with squamous cell carcinoma due to the association

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with pulmonary hemorrhage or the lack of effectiveness, respectively.[3,4] Finally, two therapeutically targetable alterations, *DDR2* mutation[5] and *FGFR1* amplification,[6] have recently been identified in pulmonary squamous cell carcinoma. These advances underlie the growing importance of accurate identification of non-small cell carcinoma subtype for assigning patients to appropriate 'histology-based' therapies and the triage of tissue for appropriate molecular studies.

Despite the clear need for pathologists to subclassify non-small cell carcinomas accurately, making the morphological distinction between squamous cell carcinoma and adenocarcinoma can be difficult. Distinguishing features, such as gland formation or keratinization, are not always evident in poorly differentiated carcinomas, particularly when the material for review is scant (eg, biopsy or cytology). Numerous immunohistochemical markers have been recently explored for their utility in distinguishing pulmonary squamous cell carcinoma and adenocarcinoma, and p63 has emerged as the 'front runner' of the squamous markers.[7–11] Several studies have shown that p63 has an extremely high sensitivity (approaching 100%) for squamous cell carcinoma.[7–9,11–15] However, the main limitation of p63 is low specificity due to its unexpected reactivity in 16–65% of lung adenocarcinomas.[2,9,14,16–18] In the majority of p63-positive adenocarcinomas, expression is focal, but in a subset, it is strong and diffuse, approaching the extent typical of squamous cell carcinoma.[9,12,14,16] Another important limitation of p63 as a 'squamous marker' is its unexpected expression in several other tumor types, particularly lymphomas, where reactivity has been reported in up to half of the cases.[19–24] This is a particularly treacherous pitfall with drastic treatment implications, as large cell lymphoma may present as a solitary thoracic mass, and its epithelioid morphology may closely mimic non-small cell carcinoma.[25–27] In this setting, strong expression of p63 could misclassify an unsuspected lymphoma as squamous cell carcinoma. Clearly, a marker of squamous differentiation that shares the sensitivity of p63, but is also highly specific, would be extremely useful.

p63 is normally expressed in the basal or progenitor cell layer of stratified epithelia (eg, squamous, urothelial, bronchial), basal cells of some glandular epithelia (eg, prostate), as well as myoepithelial cells of breast and salivary glands, trophoblasts and thymic epithelial cells. Tumors consistently positive for p63 include squamous cell carcinomas of lung and extra-pulmonary sites, urothelial, myoepithelial, trophoblastic, and thymic epithelial neoplasms—the malignant counterparts to cells normally expressing p63. The perplexing issue with p63 is its unexpected sporadic presence in various other tumor types, such as lung adenocarcinomas and lymphomas, mentioned above, as well as some sarcomas and various carcinomas, eg ovarian, endometrial, breast, and colorectal.[28,29]

Although frequently thought of as a single molecule, p63 in fact consists of several variants (isoforms), which fall into two major groups—TAp63 and ΔNp63 (Figure 1). These isoforms differ in the structure of the N-terminal domain. TAp63 isoform contains a transactivation-competent 'TA' domain with homology to p53, which regulates expression of the growth-inhibitory genes. On the other hand, ΔNp63 isoform contains an alternative transcriptionally-inactive 'ΔN' domain, which is thought to antagonize the activity of TAp63 and p53.[30] Therefore 'p63' is a 'two-in-one' family of opposing molecules: TA—a p53-like tumor suppressor and ΔN—an oncogene. It has been suggested primarily in laboratory studies that the predominant p63 isoform in basal/progenitor cells is specifically the ΔN variant, whereas the TA isoform has a wider tissue distribution.[28,29,31] ΔNp63 is thought to function as a stem cell factor, responsible for maintaining cells in an uncommitted state with regenerative potential—a role that may be recapitulated in tumors derived from these cells.[31,32] In line with this functional role, it was noted that the predominant p63 transcript in squamous cell carcinomas of lung and other sites is ΔNp63.[33,34] As a corollary, these studies suggest that it is the TAp63 isoform that is responsible for the unexpected presence of p63 in certain tumors.

The p63 antibody that is routinely used in most pathology laboratories is 4A4. As shown in Figure 1, this antibody recognizes both TAp63 and ΔNp63 isoforms, and it is therefore a 'pan-p63' marker. Antibodies that distinguish different p63 isoforms, particularly the antibody designated p40 which recognizes exclusively ΔNp63 and not TAp63, have been available for several years.[35] Surprisingly, p40 antibody is virtually unknown as a diagnostic marker in pathology, although its use has been

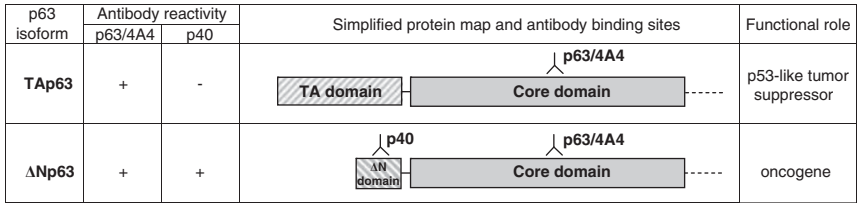

Figure 1 Diagram of p63 isoforms and antibody binding sites.

reported in a several organ systems, including squamous cell carcinomas of head and neck,[35] and esophagus,[36] thymomas,[19] urothelial carcinomas,[37] and trophoblastic tumors.[38] Recently, several studies have reported on the use of p40 for the distinction of lung squamous cell carcinoma and adenocarcinoma, suggesting that unlike p63 4A4 antibody, p40 antibody is highly squamous-specific.[13,39–41] Here we sought to expand on these initial observations and to comprehensively compare the performance of p40 (antibody recognizing ΔNp63 only) *versus* p63/4A4 (antibody recognizing both ΔNp63 and TAp63 isoforms) in a large series of whole tissue sections of lung squamous cell carcinoma and adenocarcinoma ($n=318$). In addition, given the potential pitfall in differentiating unsuspected p63-positive large cell lymphomas from squamous cell carcinoma, we evaluated the reactivity of p40 *versus* p63 in various large cell lymphomas ($n=152$), specifically focusing on the types which may present as a solitary thoracic mass and feature epithelioid morphology (diffuse large B-cell lymphoma, mediastinal large B-cell lymphoma, anaplastic large cell lymphoma).

Materials and methods

Study Design and Tumor Description

Study approval was obtained from the Institutional Review Board of Memorial Sloan–Kettering Cancer Center, New York, NY, USA, and The Johns Hopkins Hospital, Baltimore, MD, USA. Whole-tissue sections of resected squamous cell carcinomas ($n=81$) and adenocarcinomas ($n=237$) were selected from of the previously published series of non-small cell carcinomas from Memorial Sloan–Kettering Cancer Center[9] and The Johns Hopkins Hospital,[14] in which immunohistochemical characterization of these tumors with p63 4A4 antibody was reported. We reanalyzed p63 immunohistochemistry and performed immunohistochemistry with p40 antibody for all squamous cell carcinomas ($n=81$) and a subset of adenocarcinomas ($n=205$). The scoring of p40 immunoreactivity was performed without the knowledge of p63 reactivity. Cases were included if there was at least focal unequivocal squamous or glandular (but not both, ie, adenosquamous) morphological differentiation. The tumors were enriched for moderately- and poorly-differentiated carcinomas, as well-differentiated tumors would be unlikely to cause diagnostic difficulty in small samples. The carcinomas were graded by standard criteria.[42] The squamous cell carcinomas consisted of 36 (44%) moderately and 45 (56%) poorly differentiated tumors. Among adenocarcinomas, there were 25 (11%) well-, 125 (53%) moderately-, and 87 (37%) poorly differentiated carcinomas.

Large cell lymphomas were evaluated using tissue microarrays that were constructed using specimens retrieved from the surgical pathology archives of the Memorial Sloan–Kettering Cancer Center. Included were 73 primary mediastinal large B-cell lymphomas, 67 diffuse large B-cell lymphomas not otherwise specified, and 12 anaplastic large-cell lymphomas. The tissue was obtained from routine formalin-fixed (10% buffered formalin), paraffin-embedded blocks, and three core samples were taken from each block to address tumor heterogeneity.

Immunohistochemistry

Immunohistochemistry with p40 antibody (5–17, CalBiochem/EMD Biosciences, Cat No PC373, 1:2000 dilution) was performed at the Memorial Sloan–Kettering Cancer Center on Ventana Discovery XT automated stainer (Ventana Medical Systems, Tucson, AZ, USA). 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 as described previously.[9,14] As illustrated in Figure 1, p63 (4A4) recognizes an epitope shared by TAp63 and ΔNp63 isoforms, whereas p40 recognizes an epitope which is unique to ΔNp63.

For all markers, both extent (% cells) and intensity (1+, 2+, 3+) of immunoreactivity were recorded. H ('histological') scores were derived by multiplying percentage of immunoreactive cells by the intensity score. Bronchial basal cells served as positive controls for p63 and p40 reactivity. Only nuclear immunoreactivity was accepted. Scoring of p63 and p40 in squamous cell carcinomas excluded overtly keratinized cells, which lose the reactivity for both markers. The *P*-values for the comparison of immunoreactivity were determined by a two-tailed Mann–Whitney test.

Results

As shown in Table 1 and Figure 2, both p63 and p40 were positive in 81/81 (100%) of squamous cell carcinomas. The reactivity for both p63 and p40 was consistently strong and diffuse; the vast majority (95%) of squamous cell carcinomas had 90–100% of tumor cells strongly labeling for both markers. The mean (range) percentage of p63 and p40 immunoreactive cells was 97% (50–100%) and 96% (50–100%), respectively. Subtotal reactivity for either marker was highly uncommon; only four cases (5%) had reactivity in 50–70% of tumor cells. The reactivity for p63 *versus* p40 was equivalent in squamous cell carcinoma as measured by the mean percentage of immunoreactive cells (97 *vs* 96%, respectively; $P=0.73$), the mean intensity score (2.9 *vs* 2.9, respectively; $P=0.98$), and the mean H score (283 *vs* 279, respectively; $P=0.77$).

Among adenocarcinomas, p63 showed reactivity in 74/237 (31%) of cases. The p63-positive adenocarcinomas included 11 well-, 40 moderately-, 23

Table 1 Immunoreactivity for p63 *vs* p40 in squamous cell carcinoma, adenocarcinoma and large cell lymphoma

Tumor type	*Ab*	N-*tested*	N *(%) of cases with the following proportion of immunoreactive tumor cells*						*Mean (range) for the following parameters among positive cases*		
			0	*1–5%*	*6–20%*	*21–50%*	*>50%*	*Any*	*Immunoreactive cells (%)*	*Intensity score*	*H score*
Squamous cell carcinoma	p63	81	0	0	0	2 (2)	79 (98)	81 (100)	97 (50–100)	2.9 (2–3)	283 (100–300)
	p40	81	0	0	0	2 (2)	79 (98)	81 (100)	96 (50–100)	2.9 (1–3)	279 (100–300)
Adenocarcinoma	p63	237	163 (69)	16 (7)	30 (13)	19 (8)	9 (4)	74 (31)	26 (1–90)	2.2 (1–3)	59 (2–240)
	p40	205	198 (97)	7 (3)	0	0	0	7 (3)	4 (1–5)	2.3 (1–3)	10 (2–15)
Large cell lymphoma–all tested types	p63	152	70 (46)	8 (5)	14 (9)	25 (16)	35 (23)	82 (54)	48 (2–100)	1.8 (1–3)	101 (1–300)
	p40	152	152 (100)	0	0	0	0	0	N/A	N/A	N/A
Diffuse large B-cell lymphoma	p63	67	34 (51)	1 (1)	8 (12)	10 (15)	14 (21)	33 (49)	49 (3–100)	1.8 (1–3)	107 (2–300)
	p40	67	67 (100)	0	0	0	0	0	N/A	N/A	N/A
Mediastinal large B-cell lymphoma	p63	73	28 (38)	6 (8)	4 (5)	14 (19)	21 (29)	45 (62)	51 (2–100)	1.8 (1–3)	104 (1–300)
	p40	73	73 (100)	0	0	0	0	0	N/A	N/A	N/A
Anaplastic large-cell lymphoma	p63	12	8 (67)	1 (8)	2 (17)	1 (8)	0	4 (33)	14 (3–28)	1.5 (1–2)	18 (5–28)
	p40	12	12 (100)	0	0	0	0	0	N/A	N/A	N/A

Abbreviations: Ab, antibody; N/A, non-applicable.

poorly differentiated tumors. p63 showed a wide range of reactivity with minimal reactivity ($\leq$5% of tumor cells) in 7% of cases, focal reactivity (6–50% of tumor cells) in 21% of cases, and diffuse reactivity (>50% of tumor cells)—the extent typical of squamous cell carcinoma—in 4% of cases. The mean (range) for percentage of p63-positive cells in adenocarcinomas was 26% (1–90%) and mean (range) for H score was 59 (2–240). Remarkably, of 205 adenocarcinomas (which included 74 p63-positive adenocarcinomas) only 7 cases (3%) showed labeling for p40, and in all cases, the extent of p40 reactivity was minimal, not exceeding 5% of tumor cells (range 1–5%; mean, 4.4% of cells staining). Figure 3 illustrates the remarkable difference in the frequency and the extent of p63 *versus* p40 reactivity in adenocarcinomas. The minimal p40 reactivity in rare adenocarcinomas was also readily distinguishable from the diffuse reactivity for p40 in squamous cell carcinomas, as reflected by the mean percentage of p40 immunoreactive cells (4 *vs* 96%, respectively; $P<0.0001$) and the H score (10 *vs* 279, respectively; $P<0.0001$). There were no apparent distinguishing morphological features of the seven adenocarcinomas with rare p40-positive cells. Three of these cases were moderately differentiated and four cases were poorly differentiated. Interestingly, three cases had a peculiar p40 and p63 reactivity in the peripheral 'basal-like' layer of tumor nests, whereas the other four cases showed reactivity in scattered cells without a discernable pattern.

Examination of large cell lymphomas revealed p63 immunoreactivity in 82 of 152 (54%) cases overall, including 45 of 73 (62%) mediastinal large B-cell lymphomas, 33 of 67 (49%) diffuse large B-cell lymphomas not otherwise specified, and 4 of 12 (33%) anaplastic large-cell lymphomas. Similar to adenocarcinomas, the percent of immunoreactive lymphoma cells varied considerably, with minimal reactivity ($\leq$5% of tumor cells) in 5% of cases, focal reactivity (6–50% of tumor cells) in 25% of cases, and diffuse reactivity (>50% of tumor cells) in 23% of cases. The mean percentage of lymphoma cells labeling for p63 was 48%, and the mean H score was 101. On the other hand, p40 was entirely negative in all 152 large cell lymphomas tested. In many cases, p40 showed a peculiar weak membranous staining pattern of unclear significance that was not seen in the carcinomas. Interestingly, similar weak membranous p40 reactivity was noted in some non-neoplastic lymphocytes.

The above data is summarized in Figure 4, which highlights that p40 immunoreactivity above minimal (5%) was entirely specific for squamous cell carcinoma, and was not seen in any adenocarcinomas or large cell lymphomas, whereas p63 reactivity was seen in 24% of adenocarcinomas and 49% of lymphomas, of which a subset of cases (4% of adenocarcinomas and 23% of lymphomas) displayed diffuse p63 immunoreactivity at the level typical of squamous cell carcinomas. Examples of p63 *versus* p40 reactivities in the studied tumor types are illustrated in Figures 5 and 6.

As shown in Table 2, the sensitivity and negative predictive values of both p63 and p40 for squamous cell carcinoma were 100%. Among all the tumors tested (ie, non-small cell lung cancers and lymphomas), specificity for p63 *versus* p40 was 60 *versus* 98%, respectively. In analysis restricted to non-small cell carcinomas, specificity for p63 *versus* p40 was 69 *versus* 97%, respectively. If reactivity of $\leq$5% was disregarded, the specificity was still only 66% for p63, but 100% for p40.

Lastly, we compared the extent of p63 and p40 reactivity in squamous cell carcinoma as a function

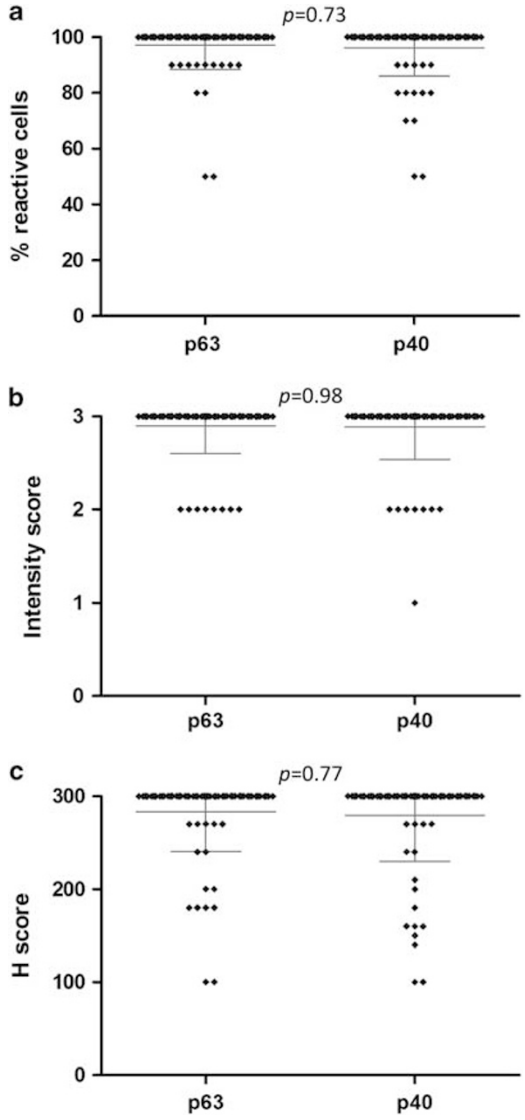

Figure 2 Equivalent extent and intensity of p63 *versus* p40 immunoreactivity in squamous cell carcinoma. Scatter dot plots for percentage of immunoreactive cells (**a**), intensity score (**b**), and H score (**c**) in 81 squamous cell carcinomas. Each dot represents reactivity in a single case. Lines and error bars indicate the mean and s.d., respectively.

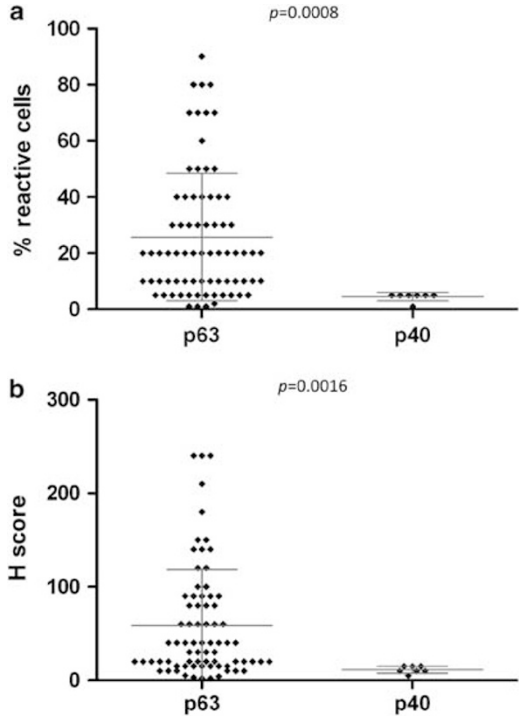

Figure 3 Comparison of p63 *versus* p40 immunoreactivity in adenocarcinoma. Scatter dot plots for percentage of immunoreactive cells (**a**) and H score (**b**) for all adenocarcinomas with p63 ($n=74$) or p40 ($n=7$) reactivity. Each dot represents reactivity in a single case. Lines and error bars indicate the mean and s.d., respectively.

of tumor grade. As shown in Figure 7, similar to p63, reactivity for p40 did not change significantly in moderately *versus* poorly differentiated carcinomas as measured by overall percentage of immunoreactive tumor cells ($P=0.42$) or H score ($P=0.44$).

Discussion

The findings in this study confirm and expand upon several recent reports, suggesting that p40 antibody (detecting ΔN isoform of p63) is markedly superior to the standard p63 4A4 antibody (detecting both ΔN and TA isoforms) in the diagnosis of pulmonary squamous cell carcinoma. The key finding in this study is that in a large series of whole-tissue sections, p40 was equivalent to p63 in sensitivity for squamous cell carcinoma; all squamous carcinomas were positive for both markers, and reactivity for both markers was consistently diffuse. The extent and intensity of p40 reactivity in squamous cell carcinoma were indistinguishable from that of p63. The second key finding is that p40 was markedly superior to p63 in specificity. Although p63 showed significant reactivity in adenocarcinomas (31% of cases) and large cell lymphomas (54% of cases), only rare adenocarcinomas (3% of cases) had labeling for p40, which was always minimal ($\leq 5\%$ of tumor cells), and absolutely all lymphomas were completely negative for p40. These findings indicate that routine use of p40 in place of p63 would avoid the potential pitfall in differentiating pulmonary squamous cell carcinoma from adenocarcinoma and large cell lymphoma based on unexpected p63 reactivity, with no compromise in sensitivity.

Overall our findings confirm the initial observations made in several smaller studies in which immunohistochemistry for p40 was evaluated in lung adenocarcinomas and squamous cell carcinomas. The first study to describe immunohistochemistry with p40 antibody was by Hibi *et al*[35] in 2000, where p40 was found to be entirely sensitive and specific for squamous cell carcinoma, based on

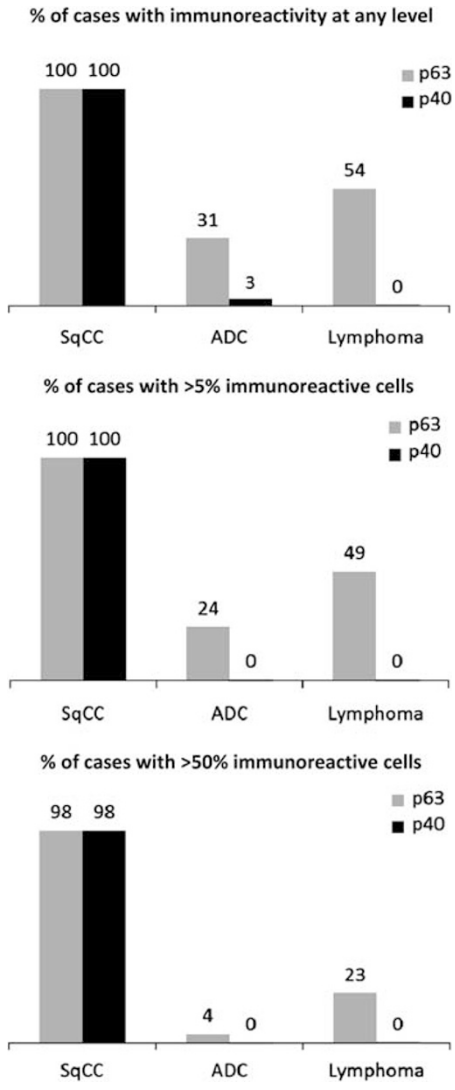

Figure 4 Summary for p63 *versus* p40 at different thresholds of reactivity. SqCC, squamous cell carcinoma; ADC, adenocarcinoma.

analysis of 23 lung carcinomas. This finding, however, has remained dormant until recently, when Pelosi *et al*[39] examined whole-tissue sections of 20 pulmonary adenocarcinomas, and subsequently 46 matched small-biopsy/cytology samples and surgical resections of non-small cell carcinomas,[40] and found p40 antibody, unlike p63 4A4 antibody, to be 100% squamous-specific. Also recently, Righi *et al*[41] found p40 to have 100% sensitivity and 97% specificity for the diagnosis of squamous cell carcinoma, based on 57 cytology specimens. In the one case of p40 labeling in adenocarcinoma, the staining was described as weak and focal. Finally, Del Vescovo *et al*[13] investigated p40 reactivity in 50 lung resections, and found it to be 95.8% sensitive and 100% specific for squamous cell carcinoma. Although the remarkable specificity of p40 compared with p63 was suggested in these prior investigations, the main contribution of the current study is that here we performed a comprehensive comparison of p63 and p40 in a large series of whole-tissue sections of lung carcinomas ($n = 318$), allowing us to establish definitively the markedly superior specificity and equivalent sensitivity of p40 compared with p63 for squamous cell carcinoma. In particular, our conclusion regarding the specificity was based on analysis of a total of 74 p63-positive adenocarcinomas, drawn from collections from two large academic institutions.

Given the exceedingly high specificity of p40 for squamous cell carcinoma, the significance of rare adenocarcinomas with p40 immunoreactive cells (which in all cases was minimal, not exceeding 5% of tumor cells) is unclear. These adenocarcinomas labeled diffusely for TTF-1 and there was no overt morphological evidence of squamous differentiation (data not shown). Additional studies will be needed to investigate the potential significance of p40 reactivity in these rare adenocarcinomas, particularly in tumors with a peculiar basal-like distribution of immunoreactive cells. From a practical standpoint, because of minimal extent of p40 reactivity (compared with consistently diffuse positivity in squamous cell carcinoma) and diffuse expression of TTF-1 (which is not expected for squamous cell carcinoma), this reactivity should not present a difficulty in the distinction from squamous cell carcinoma. Of note, in this series we have not encountered focal but significant p40 reactivity (10–40%) in either adenocarcinoma or squamous cell carcinoma; therefore the interpretation of such reactivity, if encountered in a clinical setting, needs further study.

It is interesting to note that frequent p63 reactivity and virtual absence of p40 reactivity in lung adenocarcinomas appears to parallel the pattern for these markers in reactive pneumocytes in non-neoplastic pulmonary conditions. It was previously noted that reactive pneumocytes may occasionally label for p63,[43,44] whereas in a study of usual interstitial pneumonias, Chilosi *et al*[43] found that although p63 occasionally labeled reactive type II pneumocytes, these cells were consistently negative for p40, indicating that this reactivity was due to the expression of the TA rather than ΔN isoform of p63. Thus, the reactivity for p63, but not p40 of lung adenocarcinoma, appears to recapitulate the biological properties of pneumocytes in reactive conditions.

In addition to the analysis of p40 *versus* p63 in non-small cell carcinomas, this study represents the largest review of these markers in various large cell lymphomas. This is relevant because occasionally these tumors can be mistaken for lung cancer, and p63 reactivity in this setting can be misleading. Anecdotally, a case submitted to one of our institutions in consultation as suspected squamous cell carcinoma on the basis of p63 labeling was later proven to be lymphoma on further workup (WDT and NR, unpublished observations). p63 labeling in

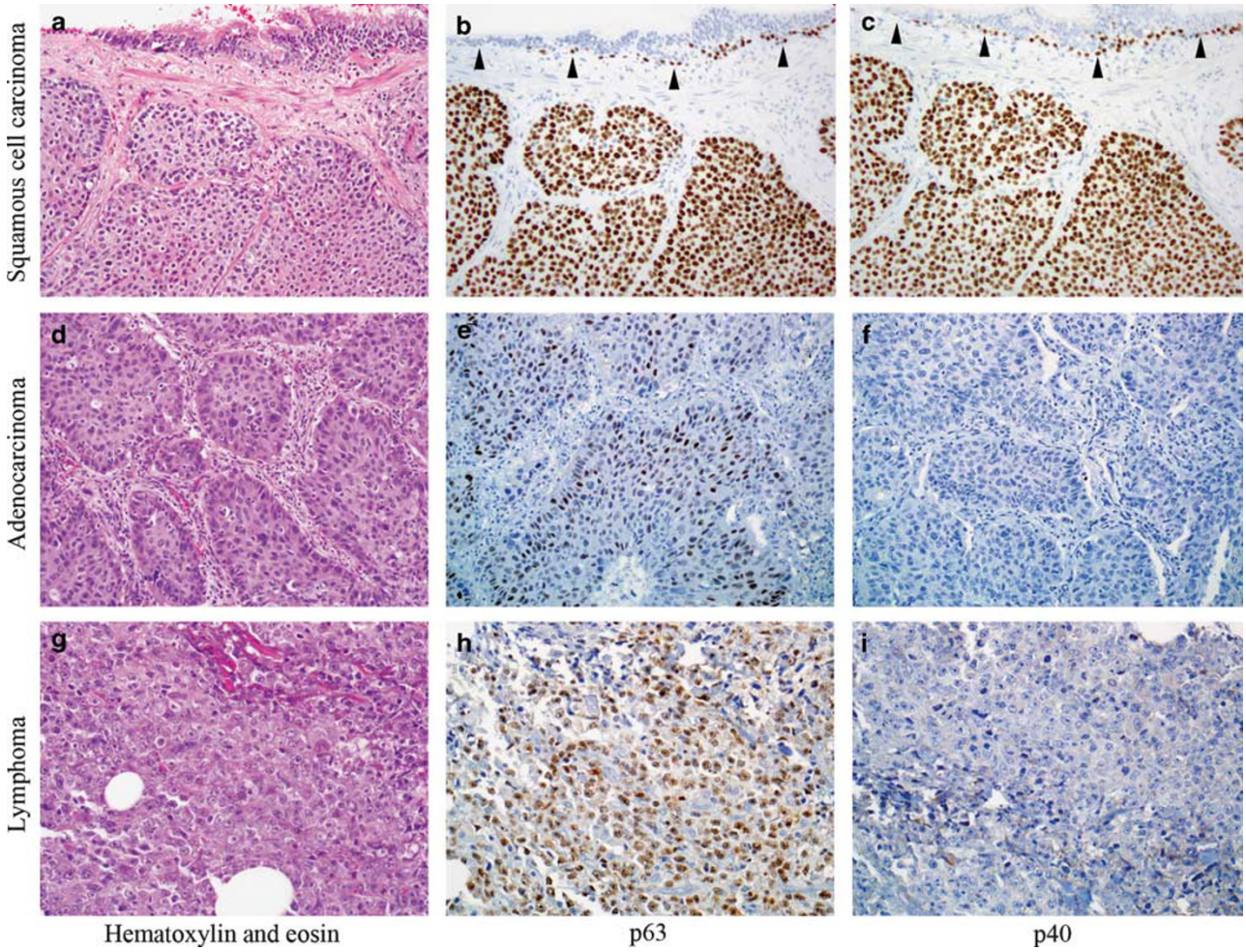

Figure 5 Examples of p63 *versus* p40 reactivity in squamous cell carcinoma, adenocarcinoma, and large cell lymphoma. (**a–c**) This poorly differentiated squamous cell carcinoma does not have overt squamous differentiation in this field (**a**, hematoxylin and eosin, ×200), but p63 (**b**, p63 immunohistochemistry, ×200), and p40 (**c**, p40 immunohistochemistry, ×200) are strongly and diffusely positive. Arrowheads indicate bronchial basal cells, which label equivalently for p63 and p40. (**d–f**) Although there were areas of clear-cut glandular differentiation elsewhere, in this field it is difficult to distinguish this poorly differentiated adenocarcinoma from a squamous cell carcinoma (**d**, hematoxylin and eosin, ×200). Although p63 is diffusely positive with moderate to strong intensity (**e**, p63 immunohistochemisty, ×200), p40 is entirely negative (**f**, p40 immunohistochemistry, ×200). **(g–i)** This diffuse large B-cell lymphoma has a very epithelioid appearance (**g**, hematoxylin and eosin, ×400). In addition, this lymphoma is strongly and diffusely immunoreactive for p63 (**h**, p63 immunohistochemisty, ×400). p40 is, however, completely negative (**i**, p40 immunohistochemistry, ×200).

large cell lymphomas in our study is in the range of what has been previously reported.[19,20,22–24,28,45] Furthermore, several prior studies have noted that this reactivity was attributable to TAp63 rather than ΔNp63 isoform at mRNA level[22,28] and by immunohistochemistry,[45,46] and our findings confirm these observations. The functional significance of frequent p63 reactivity in lymphomas is unclear, but it may be related to p63 (but not p40) occasionally labeling germinal center lymphocytes.[21,22] Although the propensity of large cell lymphomas to label for p63 is well documented, the fact that this represents an important pitfall in the differential diagnosis with squamous cell carcinoma has received little attention in the literature. This is an important consideration in the lung, as lymphomas we have examined may occasionally present as a solitary thoracic mass, clinically mimicking a much more common carcinoma. In particular, mediastinal large B-cell lymphoma may be confused radiologically with a centrally-located squamous cell carcinoma.[27] Furthermore, these lymphomas may have a remarkably epithelioid histological appearance, simulating a non-small cell carcinoma.[25,26] Certainly, additional markers can readily confirm the diagnosis of lymphoma if this diagnosis is suspected (eg, CD45, CD20, ALK, CD30, and negative cytokeratins). However, diffuse reactivity for p63 in an unsuspected large cell lymphoma may represent a critical pitfall, leading to misinterpretation as squamous cell carcinoma. This particularly applies to anaplastic large-cell lymphomas, which may be negative for CD45 and positive for EMA.[26]

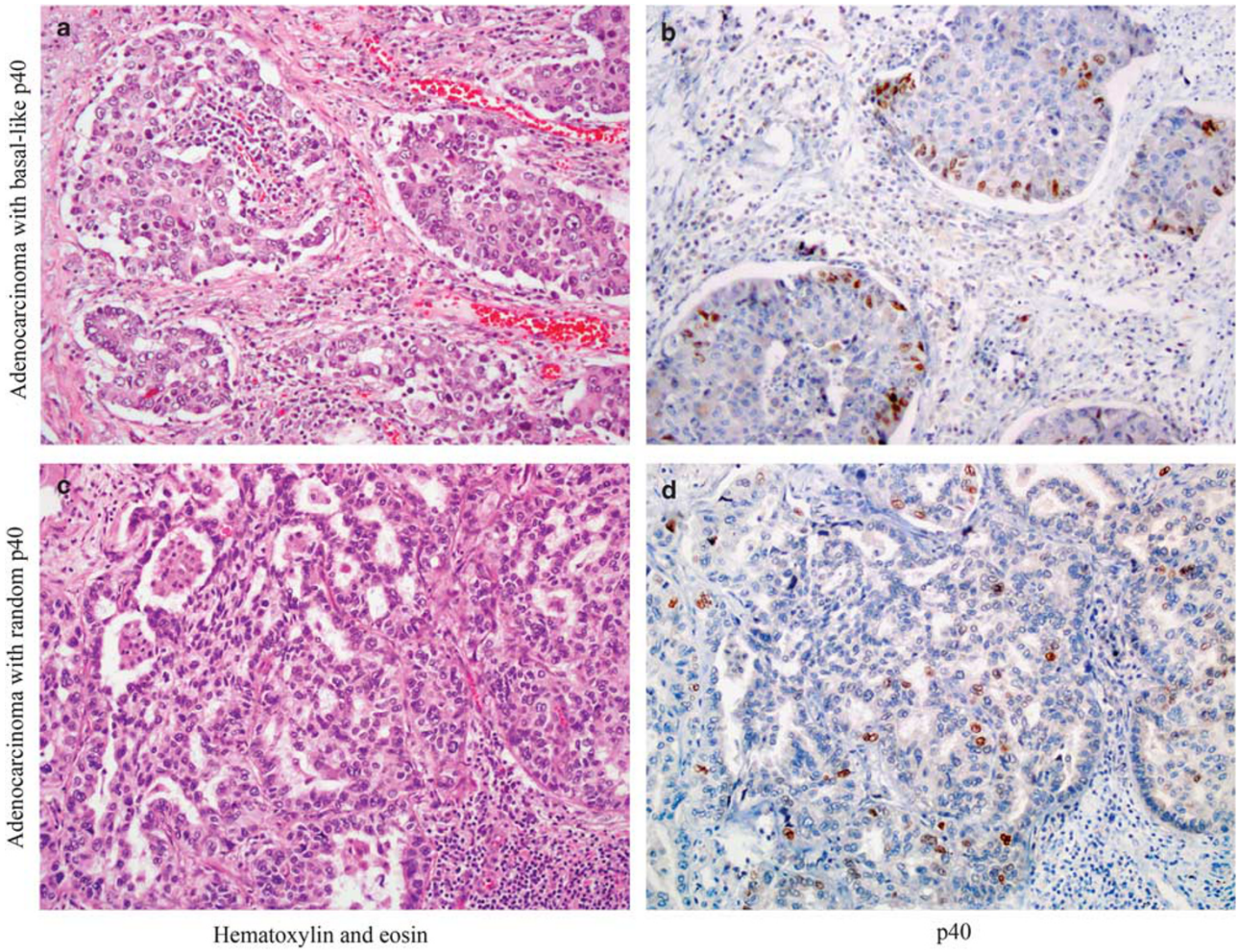

Figure 6 Examples of rare adenocarcinomas with focal p40 immunoreactivity. (**a**, **b**) Rare adenocarcinoma with basal-like distribution of p40. An area with the most prominent reactivity is illustrated (**a**, hematoxylin and eosin, ×200; **b**, p40 immunohistochemistry, ×200). (**c**, **d**) Rare adenocarcinoma with p40 labeling in scattered cells in a random distribution (**c**, hematoxylin and eosin, ×200; **d**, p40 immunohistochemistry, ×200). Either pattern is readily distinguishable from the diffuse p40 reactivity in squamous cell carcinoma.

Table 2 Sensitivity and specificity of p63 *vs* p40 for squamous cell carcinoma

	Unquantified reactivity[a]				*>5% Reactivity*[b]			
	Sensitivity (%)	*Specificity (%)*	*PPV (%)*	*NPV (%)*	*Sensitivity (%)*	*Specificity (%)*	*PPV (%)*	*NPV (%)*
p63	100	60	34	100	100	66	38	100
p40	100	98	92	100	100	100	100	100

Abbreviations: PPV, positive predictive value; NPV, negative predictive value.
[a]Data for any amount of reactivity considered as positive.
[b]Data for reactivity above minimal (>5%) considered as positive.
[a,b]Data for squamous cell carcinoma *vs* non-squamous tumors (adenocarcinoma+lymphoma).

In addition to p63, numerous other squamous markers, including CK5/6, 34βE12, Desmocollin-3, S100A2, S100A7, SOX2, Glypican 3, and microRNA miR-205, have been recently evaluated for the ability to distinguish pulmonary squamous cell carcinoma from adenocarcinoma.[8,9,11,14,17,47] None of these markers have a sensitivity/specificity profile that matches that of p40. The only other marker with reported specificity near 100% for squamous cell carcinoma is Desmocollin-3. However, its reported sensitivity has ranged from 52–100%.[17,41,47] Furthermore, p40 is a nuclear marker, which is less prone to potential non-specific reactivity and difficulty in interpretation as can happen with

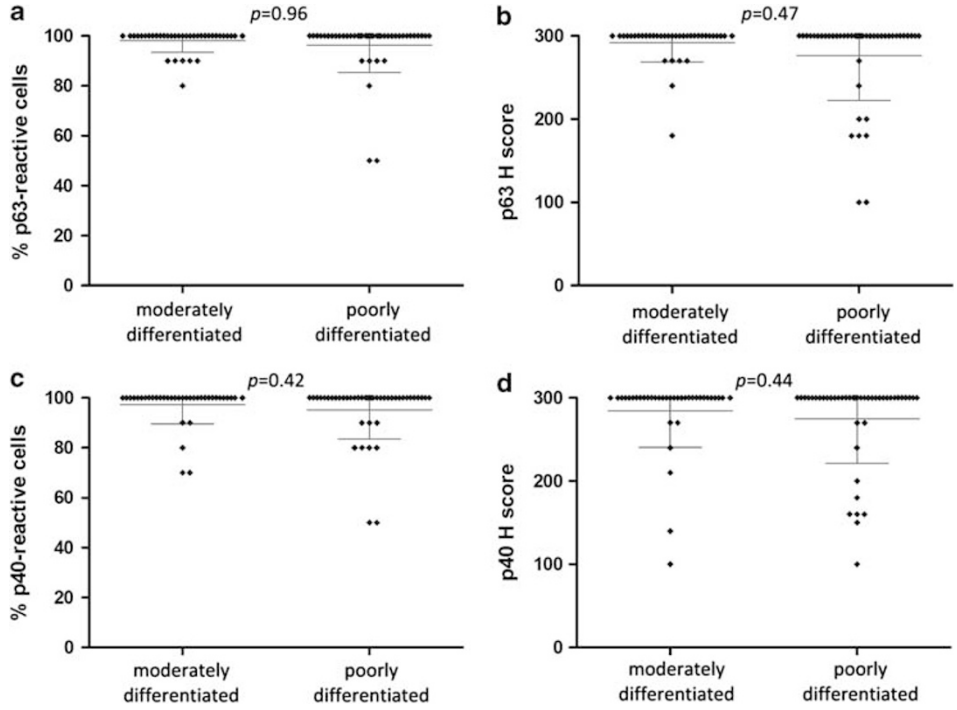

Figure 7 Reactivity for p63 and p40 is similar in moderately *versus* poorly differentiated squamous cell carcinomas. Scatter dot plots for p63 *versus* p40 reactivity in moderately differentiated ($n=36$) *versus* poorly differentiated ($n=45$) squamous cell carcinomas by percentage of immunoreactive cells (**a**, **b**) and H score (**c**, **d**). Each dot represents reactivity in a single case. Lines and error bars indicate the mean and s.d., respectively.

cytoplasmic markers like Desmocollin-3. Although direct comparison in a single study is warranted, our findings suggest that p40 is currently the best marker for squamous cell carcinoma. Because of its excellent sensitivity and specificity profile, p40 is the first lung marker with practically a dichotomous interpretation for non-small cell carcinoma subtyping: diffuse labeling supports squamous cell carcinoma, whereas the absence of reactivity is a strong argument against this diagnosis.

The optimal panel of immunohistochemical markers for non-small cell lung carcinoma subtyping, with a focus on small specimens, has been evaluated in several recent studies.[8,9,11,41,48,49] In most studies, the suggested marker panels included TTF-1 and p63 with variable addition of CK5/6 (as additional squamous marker) and Napsin A +/− mucin stains (as additional glandular markers). We (NR, WDT) recently suggested that a combination of TTF-1 and p63 is sufficient to determine the tumor type in the majority of adenocarcinomas and squamous cell carcinomas.[9] Despite its low specificity, we previously showed that p63 performs well if interpreted in the context of TTF-1.[9] As p63 4A4 is a well-established antibody in most pathology laboratories, it is reasonable to ask what advantage (other than the lack of reactivity in lymphomas described above) can be offered by p40 compared with p63. One limitation of the prior two-marker algorithm is that a subset of cases still requires the addition of CK5/6 to confirm squamous-specific expression of p63. However, CK5/6 has an imperfect sensitivity and specificity.[9,17] Furthermore, minimizing the number of immunostains is an important consideration given the need to conserve tissue for potential molecular studies (ie, *EGFR*, *KRAS*, *EML4-ALK*).[50] Another limitation of the prior algorithm is the dependence of p63 interpretation on TTF-1: only diffuse p63 labeling in the absence of TTF-1 is accepted as an indicator of squamous differentiation, whereas p63 reactivity (even if diffuse) in a TTF-1-positive carcinoma is interpreted as nonspecific. However, this means that an unsuspected technical failure of TTF-1 immunostaining could lead to an erroneous diagnosis of squamous cell carcinoma. This is an especially important consideration in small specimens, where internal control cells may not be represented and, unlike whole tissue sections, technical failure may not be apparent. Furthermore, it is well documented that TTF-1 (specifically the SPT24 clone) can rarely show focal reactivity in squamous cell carcinomas,[51] further complicating the dependence of p63 interpretation on TTF-1 status. p40 therefore offers a significant advantage over p63 in that its interpretation does not require CK5/6 as additional squamous marker and does not depend on TTF-1. The performance p40 in small specimens will need further validation, but excellent results have already been suggested in small biopsy and cytology samples in several laboratories.[13,40,41]

Overall, p63 4A4 is one of the most widely utilized multipurpose antibodies in the diagnostic immunohistochemistry of tumors. Common applications outside of lung include the diagnosis of invasion in prostate and breast cancer, where p63 is used to document the loss of basal and myoepithelial cells, respectively, and the diagnosis of squamous carcinomas of various sites, as well as urothelial and myoepithelial neoplasms.[30] Unexpected p63 reactivity in large cell lymphomas and various other tumor types may also present a diagnostic dilemma in these extra-pulmonary settings, and it may therefore be of interest to explore the utility of p40 at other sites.

In conclusion, we find that p40 is equivalent to p63 in sensitivity for pulmonary squamous cell carcinoma, but it has a marked advantage over p63 in that it is also remarkably specific. 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. 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.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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