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Original Article

Improved lung cancer classification using new optimized immunohistochemical assay with anti-p40 (BC28) mouse monoclonal antibody

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Abstract: Background: The precise differentiation of lung adenocarcinoma (ADC) and lung squamous cell carcinoma (SCC) is essential for the determination of appropriate cancer therapy. A major limitation of the currently used anti-p63 (4A4) antibody is low specificity due to its reactivity in ADCs. The anti-p63 antibody recognizes two p63 isoforms-long TAp63 isoform and a truncated p40 isoform, while the new anti-p40 (BC28) antibody recognizes only the short p40 isoform. The goal of this study was to compare the performance of the new optimized assay using anti-p40 (BC28) antibody with the VENTANA anti-p63 (4A4) antibody and with the commercially available VP Echelon™ Series p40 assay (Biocare Medical Inc.). Methods: Formalin-fixed, paraffin-embedded (FFPE) tissues from 538 patients with various lung tumors were evaluated for p40 and p63 expression by IHC using recommended protocols. Results: The new optimized assay using anti-p40 (BC28) antibody demonstrated significantly increased specificity for ADC classification compared to the anti-p63 (4A4) antibody; 10.8% lung ADCs (23/212) were positive with anti-p63 antibody but only 4.9% (11/212) ADCs were positive with anti-p40 antibody. Furthermore, the new p40 assay showed significantly superior sensitivity to the p40 Echelon™ Series assay in SCC classification; 92.6% of cases (175/189) showed p40 positive staining with the new p40 assay compared to 75.1% SCC cases (142/189) stained with VP Echelon™ Series p40 assay. Conclusions: In summary, our data indicate that the optimized p40 assay demonstrate an increased specificity compared to IHC assay using anti-p63 (4A4) and increased sensitivity over the VP Echelon™ p40 assay.

Keywords: Lung squamous cell carcinoma, lung adenocarcinoma, anti-p40 antibody, anti-p63 antibody

Introduction

Lung cancer is the most common cause of cancer-related mortality in the world [1]. Two main types of lung cancer are small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). NSCLC can further be divided into the several subtypes including squamous-cell carcinoma (SCC), adenocarcinoma (ADC), and large-cell carcinoma. Importantly, NSCLC accounts for about 85% of newly diagnosed lung cancer cases [2].

The precise differentiation of lung ADC and lung SCC is essential for the determination of appropriate cancer therapy. For example, bevacizumab shows beneficial outcome for non-squamous NSCLC, but it is contraindicated in SCC because of bleeding complications including potentially fatal pulmonary hemorrhage [3]. Another example includes Gefinitib that is effective in lung ADC but not in SCC [4]. Therefore, there is a high demand to employ specific tumor markers for reliable identification of the lung cancer subtype, especially in poorly differentiated tumors or small biopsies.

One of the most frequently recommended assays for the differentiation of lung ADC and SCC is immunohistochemistry (IHC) with anti-p63 antibody, which has high sensitivity for lung squamous cell carcinoma [5, 6]. However, a limitation of this antibody is low specificity for lung adenocarcinoma [7]. Other markers used for the differentiation of lung tumors include thyroid transcription factor (TTF1) and Napsin

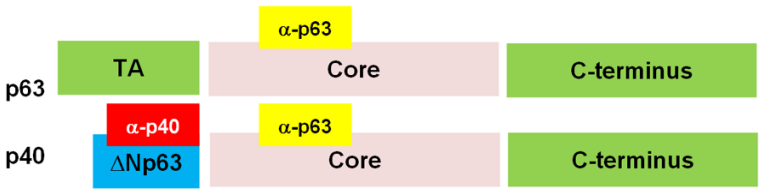

Figure 1. Scheme of the p63 and p40 (ΔNp63) proteins. (α-p40 = anti-p40 antibody, α-p63 = anti-p63 antibody, TA = transactivation).

A, which are specific for lung adenocarcinoma, and high molecular weight cytokeratins (CK) such as CK5/6, which is a specific marker of lung squamous cell carcinoma.

The anti-p63 antibody (clone 4A4) recognizes two isoforms of the p63 gene product-a longer TAp63 isoform that contains the N-terminal transactivation domain and acts as a tumor suppressor and a truncated variant designated as p40 (ΔNp63), that is lacking the N-terminal domain and acts as an oncogene (**Figure 1**). In contrast, the new anti-p40 antibody (clone BC28) recognizes only the p40 (ΔNp63) isoform but not the TAp63 isoform [7, 8]. Previous studies have shown that the anti-p40 antibody is superior to the p63 antibody since it is more specific for lung adenocarcinoma [7, 9] and thus the anti-p40 antibody has been recommended instead of the anti-p63 antibody for the diagnosis of pulmonary squamous cell carcinoma [7].

The primary goal of this study was to compare a newly developed immunohistochemical assay using the anti-p40 (BC28) mouse monoclonal antibody [referred to as anti-p40 (BC28) antibody] with 1) the commercially available VP Echelon™ Series with p40 (M) prediluted monoclonal antibody (referred to as VP Echelon™ Series p40 assay), and 2) an IHC assay using VENTANA anti-p63 (4A4) mouse monoclonal primary antibody [referred to as anti-p63 (4A4) antibody] in various lung tumors. The VP Echelon™ Series p40 assay was developed by Biocare Medical and employs the same antibody clone (BC28) as a new assay developed by Ventana Medical Systems, Inc. Tissue arrays containing different lung tumors were used in these studies. In addition, because of the importance of pre-analytical standardization, the effect of different fixatives, the duration of fixation and the fixation delay were evaluated in a squamous cell carcinoma xenograft model.

Materials and methods

Materials

The human CaSki squamous cell carcinoma cell line was obtained from American Type Culture Collection (ATCC; Rockville, MD). The cells were cultured in RPMI-1640 medium (ATCC) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in 5% CO_2. All other chemicals were of the highest purity available.

Tumor specimens

Tissue array slides containing 538 formalin-fixed paraffin embedded (FFPE) lung cancer cases obtained from US Biomax Inc. (Rockville, MD) were evaluated for p40 and p63 expression using different IHC assays on the VENTANA BenchMark ULTRA or XT platform. The initial classification of these tumors was based on H&E staining (US Biomax Inc.). However, when H&E and p40/p63 status did not correspond with SCC/ADC classification, anti-CK 5/6, anti-Napsin A or anti-TTF-1 antibodies were used in this study for the evaluation of the discordant cases.

Optimized immunohistochemical assay using anti-p40 (BC28) antibody

The immunohistochemical method using anti-p40 (BC28) antibody was developed at Ventana Medical Systems, Inc. and optimized for the BenchMark automated stainers (ULTRA, XT, GX). The testing was performed on a Benchmark XT or ULTRA instruments with Cell Conditioning 1 for 32 min, pre-primary peroxidase inhibition and primary antibody incubation for 16 minutes. Final concentration of the anti-p40 (BC28) antibody was ~0.4 µg/ml. OptiView DAB IHC Detection Kit was used to detect p40 protein expression. To counterstain tissues the slides were incubated with Hematoxylin II and Bluing Reagent for 4 minutes. To measure the level of non-specific background signal, each slide was also stained with a mouse monoclonal antibody (MOPC-211) [Negative Control (Monoclonal), Ventana Medical Systems, Inc.] using the same protocol. This antibody is not directed against any known epitope present in human tissue. The tissues were scored as positive/negative.

Strong nuclear staining intensity was scored as a 4, strong-medium nuclear staining intensity as 3, a medium-weak nuclear staining intensity as 2, weak nuclear staining intensity as 1, and the absence of staining was scored as 0. The scores 1-4 represented positive staining, while a score less than 1 was considered negative staining.

Immunohistochemistry for p40 using VP Echelon™ Series assay for p40

The p40 expression in lung tumors was also evaluated by the commercially available VP Echelon™ Series assay using p40 (M) prediluted monoclonal antibody. The manufacturer's recommended protocol included Mild Cell Conditioning 1, primary antibody incubation for 32 minutes at 37°C, ultraBlock (BRI4001) for 4 minutes and detection with *ultra*View Universal DAB Detection kit. The slides were counterstained with Hematoxylin II for 4 minutes and Bluing reagent for 4 minutes. Each slide was also stained with a mouse monoclonal antibody (MOPC-211) [Negative Control (Monoclonal), Ventana Medical Systems, Inc.] using the same protocol. The tissues were scored as positive or negative as described in the previous section.

Immunohistochemistry for p63, napsin A, TTF1 and cytokeratin 5/6

The expression of p63, TTF1, Napsin A and CK5/6 was evaluated using prediluted anti-p63 (4A4) mouse monoclonal primary antibody, anti-TTF-1 (SP141) rabbit monoclonal primary antibody, anti-CK5/6 (D5/16B4) mouse monoclonal primary antibody (all Ventana Medical Systems, Inc., Tucson, AZ) and Napsin A (MRQ-60) mouse monoclonal primary antibody (Cell Marque, Rocklin, CA) according to recommended protocols as described in the package inserts. OptiView DAB IHC detection system was used for the detection of TTF-1 and Napsin A and *ultra*View Universal DAB detection system was used for the detection of p63 and CK5/6. The tissues were scored as positive or negative as described in the previous section.

Xenografts

All studies were conducted in accordance with the Guidance for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD) and approved by Institutional Animal Care and Use Committee. A CaSki xenograft model was selected to evaluate potential variations in staining intensity with different fixation. The CaSki cell line is derived from cervical squamous cell carcinoma and expresses the p40 protein. A total of 10×10^6 CaSki cells were implanted subcutaneously into the right flank of SCID mice. When the tumor size reached about 300 mm^3 tumors were excised, divided into smaller pieces and placed in different fixatives for various time periods or kept without fixative (ischemia) for 0.5-24 hours as described below.

Fixation studies

The effect of different fixatives and fixation time and delay to fixation was evaluated using CaSki xenograft tissues. Five fixation times for each of six common fixatives were tested. The selected times represent the lower and upper ranges of clinical histology practice and the selected fixatives represent common fixatives used globally in clinical histology practice. CaSki xenograft tissues were fixed for 1 hr, 6 hr, 12 hr and 24 hr with each of the following fixatives -10% neutral buffered formalin (10% NBF, J.T. Baker, Austin, TX), zinc formalin (Anatech Ltd, Battle Creek, MI), alcohol formalin acetic acid (AFA, Electron microscopy sciences, Hatfield, PA), 95% alcohol, PREFER fixative (glyoxal, alcohol, Anatech Ltd) and Z-5 fixative (formalin, zinc, alcohol, Anatech Ltd) prior to dehydration and embedding in paraffin. Furthermore, the effect of ischemia was evaluated. In this experiment CaSki xenograft tissues were kept on the bench at room temperature for 30 min, 1 hr, 2 hr, 6 hr, and 24 hr before fixation with 10% NBF for 24 hours. Signal intensity scores (SI) were compared to a nominal reference fixation protocol (10% NBF at room temperature for 12 hours), since 12-24 hour fixation is recommended in standard practice. Due to cross-reactivity of the mouse monoclonal anti-p40 (BC28) antibody in mouse tissues, staining was only assessed in the tumor cells in the xenograft samples.

Statistical analysis

Assuming that H&E together with the panel of other IHC markers (TTF1/Napsin A and CK5/6) is the gold standard for the correct classification of lung tumors, sensitivity was calculated as a proportion of the cases which were correctly identified by IHC as lung SCC among all lung SCC cases. Specificity was calculated as a

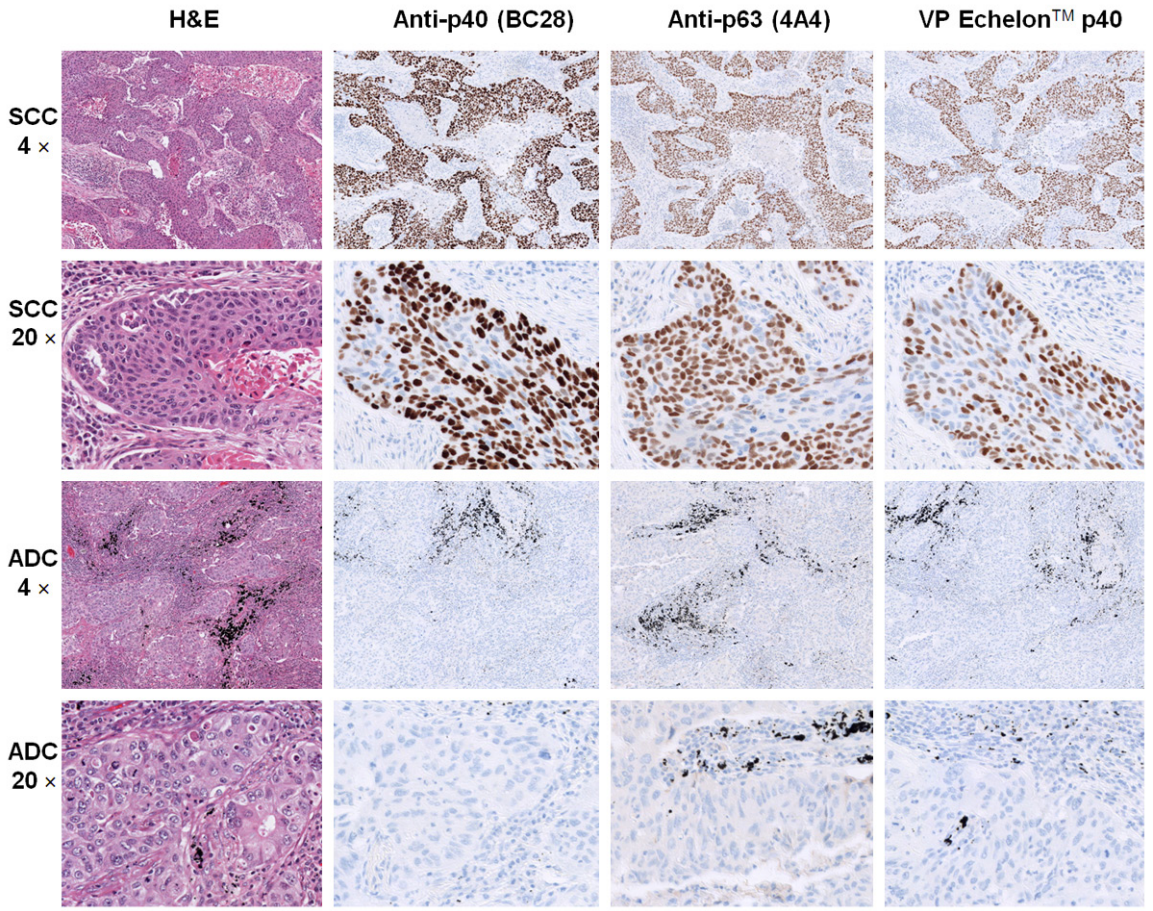

Figure 2. Images of lung squamous cells carcinoma (SCC) and lung adenocarcinoma (ADC) cases stained with H&E, anti-p40 (BC28) antibody using Ventana optimized assay, anti-p63 (4A4) antibody and VP Echelon™ Series p40 assay (4 ×, 20 ×).

proportion of the cases which were correctly identified by IHC as lung ADC among all lung ADC cases. Accuracy is the number of cases that are correctly diagnosed divided by the total number of cases. PPV is the number of true positive cases divided by the test/assay positive cases; NPV is the number of true negative cases divided by the test/assay negative cases. McNemar's exact test was used to determine *p*-values for accuracy and sensitivity. The Fleiss method was used to generate *p*-values for non-inferiority tests based off the 1-sided lower bound of the 95% confidence interval (CI) for the difference between assay specificity. All analyses were performed using SAS version 9.4 software.

Results

Lung tumors

A total of 538 lung tumors were included in the current study. Complete data sets were obtained for 464 cases successfully stained with all three assays [anti-p40 (BC28) antibody, anti-p63 (4A4) antibody and VP Echelon™ Series p40 assay]. Seventy-four cases were not compared because one or more cores fell off or were impossible to evaluate due to poor fixation, necrosis or surface chemistry issues. All of these tumors were initially classified by H&E (US Biomax). Anti-CK5/6 (D5/16B4), anti-Napsin A (MRQ-60) and/or anti-TTF-1 (SP141) antibodies were used for the evaluation of the discordant cases when H&E and p40/p63 status did not correlate. Twenty-three cases initially classified by H&E as lung SCC showed no nuclear p40/p63 staining. These were re-classified as lung ADC based on the IHC results using a lung cancer panel, which showed negative CK5/6 staining and positive staining for TTF-1 or Napsin A. One case was initially classified by H&E as lung ADC but exhibited positive signal with p40/p63. This case was reclassified as lung SCC because it was CK5/6 positive and

Table 1. Summary of the data for anti-p40 (BC28), anti-p63 (4A4) antibodies and VP Echelon™ Series p40 assay in lung SCC and lung ADC

Anti-p40 (BC28) (N = 401)	Lung SCC	Lung ADC
IHC positive	175 True positive	11* False positive
IHC negative	14 False negative	201 True negative
*10/11 cases-focal staining.		
Anti-p63 (4A4) (N = 401)	Lung SCC	Lung ADC
IHC positive	174 True positive	23* False positive
IHC negative	15 False negative	189 True negative
*16/23 cases-focal staining.		
VP Echelon™ p40 (N = 401)	Lung SCC	Lung ADC
IHC positive	142 True positive	3* False positive
IHC negative	47 False negative	209 True negative
*2/3 cases-focal staining.		

TTF-1 negative. Five cases classified as ADC and one case classified as SCC were positive for p40, p63, CK5/6 and TTF1 or Napsin A. These cases were reclassified as possible adenosquamous carcinomas. Furthermore, six cases were negative with all tested antibodies; these cases were excluded from the analysis, since it was impossible to assign them to any class.

The final distribution of the different lung cancer categories included in the study was as follows: 189 lung SCCs, 212 lung ADCs, 19 adenosquamous carcinomas (13 confirmed and 6 possible adenosquamous carcinomas), 13 bronchioloalveolar carcinomas, 2 neuroendocrine carcinomas, 6 large cell carcinomas, 6 lung carcinoids and 11 small cell undifferentiated carcinomas.

p40 and p63 immunohistochemistry

The 458 eligible tumors were evaluated by IHC using 1) the newly developed assay with the anti-p40 (BC28) antibody, 2) VENTANA anti-p63 (4A4) antibody, and 3) VP Echelon™ Series p40 assay. All three IHC assays showed specific nuclear staining with no or minimal background. Examples of the staining patterns in lung SCC and lung ADC are shown in **Figure 2**. VP Echelon™ Series p40 assay exhibited consistently less intense staining compared to optimized p40 assay (**Figure 2**).

Among 189 lung SCC, 175 tumors showed nuclear positivity for p40 with anti-p40 (BC28) antibody and 174 tumors were positive with anti-p63 (4A4) antibody. In contrast, VP Echelon™ Series p40 assay showed positive p40 staining only in 142 lung SCC cases and negative staining in 47 cases. All of these cases were confirmed to be lung SCC based on the expression of other lung cancer markers (TTF-1 or Napsin A negative, CK5/6 positive). None of the lung SCC cases were negative with anti-40 (BC28) antibody and positive with VP Echelon™ Series p40 assay. The sensitivity of anti-p40 (BC28) antibody, VENTANA anti-p63 (4A4) antibody and VP Echelon™ Series p40 assay in lung SCC was 92.6%, 92.1% and 75.1%, respectively (**Table 2**). Overall, these data indicate that new anti-p40 (BC28) assay is significantly more sensitive than the VP Echelon™ Series p40 assay in detecting lung SCC when using a McNemar's exact test ($P<0.001$). There was no significant difference between anti-p40 (BC28) antibody and the VENTANA anti-p63 (4A4) antibody sensitivity using a McNemar's exact test ($P>0.999$).

In lung ADCs, p40 and p63 immunoreactivity was uncommon. Overall, 11/212 (5.2%) lung ADC cases stained positively with anti-p40 (BC28) antibody, 23/212 (10.7%) lung ADC cases stained positively with anti-p63 (4A4) antibody, and 3/212 (1.4%) lung ADC cases stained positively with VP Echelon™ Series p40 assay (**Table 1**). The positive p40 signal was predominantly focally distributed with both IHC assays used for p40 detection (10/11 cases were focally positive with anti-p40 (BC28) antibody, 2/3 cases were focally positive with VP Echelon™ Series p40 assay, **Table 1**). In contrast, the p63 signal was diffuse in 7 cases and focal in 16 ADC cases. These data indicate that new anti-p40 (BC28) assay is more specific than the anti-p63 (4A4) antibody. The specificity of anti-p40 (BC28) antibody, anti-p63 (4A4) antibody and VP Echelon™ Series p40 assay in lung ADC was 94.8%, 89.2% and 98.6%, respectively (**Table 2**). In a non-inferiority test the specificity of the anti-p40 (BC28) antibody specificity was found to be inferior to the specificity of the VP Echelon™ p40 assay at

Table 2. Sensitivity, specificity, accuracy, positive predictive value (PPV), and negative predictive value (NPV) using Ventana p40 assay, anti-p63 (4A4) antibodies and VP Echelon™ p40 assay

	Anti-p40 (BC28) antibody	Anti-p63 (4A4) antibody	VP Echelon p40 assay
Sensitivity	92.6% (175/189)	92.1% (174/189)	75.1% (142/189)
Specificity	94.8% (201/212)	89.2% (189/212)	98.6% (209/212)
PPV	94.1% (175/186)	88.3% (174/197)	97.9% (142/145)
NPV	93.5% (201/215)	92.6% (189/204)	81.6% (209/256)
Accuracy	93.8% (376/401)	90.5% (363/401)	87.5% (351/401)

Note: All ADC cases with focal staining were included in the table.

the $\Delta = 0.05$ level ($P>0.05$). However, the specificity of the anti-p40 (BC28) antibody was found to be non-inferior to VENTANA anti-p63 (4A4) antibody at the $\Delta = 0.05$ level ($P<0.05$). **Table 2** shows the summary of the data including sensitivity, specificity, accuracy, positive predictive value (PPV) and negative predictive value (NPV).

The overall accuracy of correctly identifying lung ADCs as negative and lung SCCs as positive was significantly greater for anti-p40 (BC28) antibody p40 when compared to both assays ($P<0.05$). The accuracy of the anti-p40 (BC28) antibody, anti-p63 (4A4) antibody and the VP Echelon™ Series p40 assay was 93.8%, 90.5% and 87.5%, respectively.

The evaluation of other lung tumors revealed that among 19 lung adenosquamous carcinomas, 12 cases exhibited nuclear positivity with anti-p40 (BC28) antibody, 11 cases with anti-p63 (4A4) antibody and 5 cases with VP Echelon™ Series p40 assay (**Table 3**). One of 13 bronchioloalveolar carcinoma cases stained positively with all three IHC assays. One out of 6 large cell carcinomas showed positive focal staining with anti-p40 (BC28) antibody, two cases with VENTANA anti- p63 (4A4) antibody, and none with VP Echelon™ Series p40 assay (**Table 3**). None of the carcinoid cases, small cell undifferentiated carcinomas or neuroendocrine carcinoma cases stained positive with any assay.

The effect of fixation conditions

The results from the fixation studies showed that fixation with 10% neutral buffered formalin (NBF) for 1-24 hours was the optimal fixation condition for IHC determination of p40 expression (**Table 4**). Fixation with zinc formalin for 1-72 hours, PREFER for 6-24 hours and AFA for 1-12 hours also resulted in a good p40 signal. However, fixation with 95% EtOH did not produce equivalent p40 signal at any time point and this fixative is not suggested. In addition, the p40 staining was compromised after fixation with Z-5 compared to gold standard (10% NBF for 12 hours, **Table 4**). This fixative is not recommended.

The effect of delayed fixation on p40 expression was also studied. Xenograft tissues were left on the bench at room temperature for various time periods prior to fixation with 10% NBF for 24 hours. The results from this study show that the intensity of p40 staining is not significantly degraded after a fixation delay of up to 24 hours in CaSki xenografts.

In summary, these data suggest that 95% EtOH and Z-5 should not be used as fixatives for anti-p40 (BC28) immunohistochemistry. In addition, tissues should be fixed within 24 hours following tissue collection.

Discussion

Lung cancer is the most frequent and one of the most deadly cancer types. The most common type of lung carcinoma is NSCLC that accounts for at least 85% of all lung cancer cases in the U.S. NSCLC used to be treated as a single disease due to the similar therapeutic effects of conventional therapy. Treatment options for NSCLC included primarily surgery, radiation, and platinum-based chemotherapy [10]. However, it has been recently recognized that the different NSCLC subtypes display different patterns of genomic alterations. Furthermore, tumor histological type influences adverse effects and therapeutic response to targeted therapies such as bevacizumab [10]. Therefore, the precise classification of NSCLC is becoming increasingly important and the reliable diagnostic markers are highly needed.

The data from the present studies support the conclusions from other publications indicating that anti-p40 (BC28) antibody is sensitive and specific for the detection of p40 (ΔNp63) protein [7, 11]. The interpretation of p40 immuno-

Table 3. Summary of the staining results using Ventana p40 assay, anti-p63 (4A4) antibodies and VP Echelon™ Series p40 assay in various lung tumors

	Positive specimen % (n/N)		
Lung Cancer type	Anti-p40 (BC28) antibody	Anti-p63 (4A4) antibody	VP Echelon p40 assay
Squamous cell carcinoma (SCC)	92.6% (175/189)	92.1% (174/189)	75.1% (142/189)
Adenocarcinoma (ADC)	5.2% (11/212)	10.8% (23/212)	1.4% (3/212)
Adenosquamous carcinoma	63.2% (12/19)	57.9% (11/19)	26.3% (5/19)
Bronchioloalveolar carcinoma	7.7% (1/13)	7.7% (1/13)	7.7% (1/13)
Lung large cell carcinoma	16.7% (1/6)	33.3% (2/6)	0% (0/6)
Carcinoid	0% (0/6)	0% (0/6)	0% (0/6)
Small cell carcinoma	0% (0/11)	0% (0/11)	0% (0/11)
Neuroendocrine carcinoma	0% (0/2)	0% (0/2)	0% (0/2)

Note: For SCC, the proportions are the sensitivity of each assay; For ADC, the proportions are the specificity of each assay. All ADC cases with focal staining were included in the table.

Table 4. Summary of the fixation studies in CaSki xenografts

Fixative	Fixation period	1 hour		6 hours		12 hours		24 hours		72 hours	
	CaSki	SI	Bkg	SI	Bkg	SI	Bkg	SI	Bkg	SI	Bkg
10% NBF	Anti-p40 (BC28)	4	0	4	0	3.75*	0*	4	0	3.5	0
	Negative control	0	0	0	0	0	0	0	0	0	0
Zinc Formalin	Anti-p40 (BC28)	4	0	4	0	4	0	4	0	3.75	0
	Negative control	0	0	0	0	0	0	0	0	0	0
95% EtOH	Anti-p40 (BC28)	2	0	2	0	3	0	2.5	0	2.5	0
	Negative control	0	0	0	0	0	0	0	0	0	0
Prefer fixative	Anti-p40 (BC28)	3.5	0	4	0	4	0	4	0	3.5	0
	Negative control	0	0	0	0	0	0	0	0	0	0
Z-5 fixative	Anti-p40 (BC28)	3.5	0	3	0	3.5	0	2.5	0	3	0
	Negative control	0	0	0	0	0	0	0	0	0	0
AFA	Anti-p40 (BC28)	3.75	0	3.75	0	4	0	3.5	0	NA#	NA#
	Negative control	0	0	0	0	0	0	0	0	0	0

*Gold standard, #Tissue loss/unreadable, NA = non-applicable, NBF = neutral buffered formalin, AFA = acetic acid, formalin, alcohol; †SI = signal intensity. SI and background scores range from 0 to 4 based on a standard intensity grading.

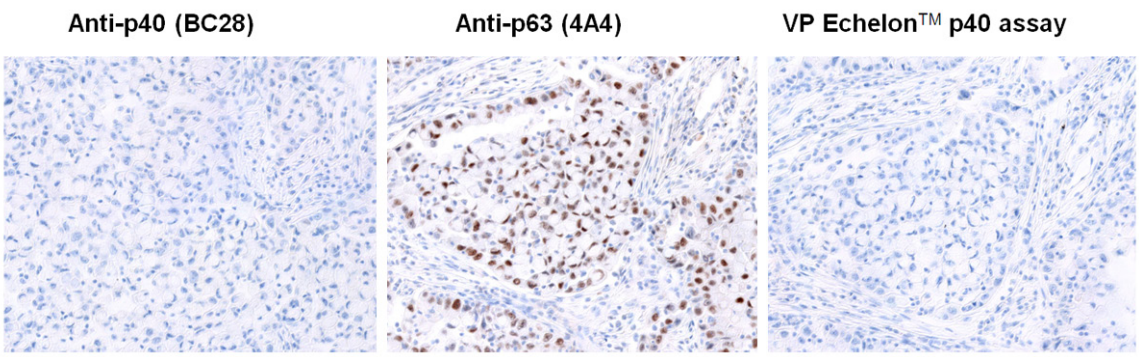

Figure 3. Image of the lung adenocarcinoma case showing diffuse positive p63 staining and negative p40 staining (20 ×).

histochemical staining was straightforward, the staining pattern was exclusively nuclear, and the background was minimal. In positive cases the staining intensity was predominantly mod-

erate to strong and occasionally weak, but it was above background levels. Infrequently weak focal staining was present in the lung ADC cases.

In addition, several previously published studies have shown that in NSCLC the anti-p40 antibody has comparable sensitivity as currently used anti-p63 antibody but higher specificity [7, 9, 12]. For example, Bishop et al. showed 100% sensitivity of anti-p40 antibody (polyclonal) and anti-p63 (4A4) antibody in SCC, however the specificities of these two antibodies were different [7]. In lung ADC, 3.4% (7/205) ADC cases showed focally positive p40 staining (specificity 96.6%), while 36.1% (74/205) ADC cases showed positive staining with anti-p63 (4A4) antibody (specificity of 63.9%) [7]. In another study, similar results were reported; the sensitivities of anti-p40 (polyclonal) antibody and anti-p63 (4A4) antibody were similar but the specificities were 96.8% and 72.8%, respectively [12].

Our study also showed that anti-p40 (BC28) antibody performs better than anti-p63 (4A4) antibody for differentiating SCC from non-SCC. The sensitivity of anti-p40 (BC28) antibody and anti-p63 (4A4) antibody was 92.6% and 92.1% for lung SCC, respectively and the specificity was 94.8% and 89.2%, respectively. In our study the anti-p63 (4A4) antibody specificity was higher than that reported in other published studies. We found 10.8% lung ADC cases staining positively with anti-p63 (4A4) antibody, 17 cases showed focal positivity and 6 cases showed diffuse staining (**Table 1**; **Figure 3**). In contrast, only 5.2% lung ADC cases stained positively with anti-p40 (BC28) antibody and all of these positive cases except one exhibited weak focal staining pattern.

The next objective of this study was to compare the new anti-p40 (BC28) assay with the VP Echelon ™ Series p40 assay. The clone of the anti-p40 antibody used in both assays is the same, however the protocols are different and the information about the diluent and the concentration of the antibody in the VP Echelon™ Series p40 assay is not available. The staining intensities with VP Echelon™ Series p40 assay were markedly lower compared to anti-p40 (BC28) antibody (**Figure 2**). Secondly, several SCC cases showing positive p63 and p40 staining were negative with VP Echelon™ Series p40 assay (**Table 1**). The specificity of VP Echelon™ Series p40 assay for lung ADC was high (98.6%) and significantly better based on non-inferiority test; the anti-p40 (BC28) assay was shown to have inferior specificity when compared to VP Echelon™ Series p40 ($P>0.05$) using the Fleiss method. However the sensitivity for lung SCC was only 75.1% and significantly worse based on McNemar's test ($P<0.05$). We speculate that the differences in the protocols, especially the OptiView DAB IHC detection kit that is used in the new assay, can be responsible for the better results with the anti-p40 (BC28) antibody.

Furthermore, using a xenograft model we have shown that optimal detection of the p40 protein by immunohistochemistry requires optimal tissue fixation with 10% neutral buffered formalin (NBF) Zinc formalin, PREFER fixative or AFA. In addition, tissues should be fixed within 24 hours following tissue collection. Other fixatives such as 95% EtOH or Z-5 may negatively affect the p40 signal intensity (**Table 4**). These data highlight the importance of correct fixation protocols in clinical practice.

Overall, the data demonstrate that anti-p40 (BC28) antibody is a useful marker to differentiate lung SCC from lung ADC. This antibody in conjunction with a panel of other key markers such as TTF-1, CK 5/6 or Napsin A can provide an accurate and reliable method for differentiating pulmonary adenocarcinoma from squamous cell carcinoma. Furthermore, this study indicates that the new Ventana assay using anti-p40 (BC28) antibody with OptiView DAB IHC detection kit is highly robust and specific and may be better suited in differentiating lung NSCLC tumors than currently used anti-p63 antibody or VP Echelon™ Series p40 assay.

Disclosure of conflict of interest

None.

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