Utilization of p40 (Δ Np63) with p63 and Cytokeratin 5/6 Immunohistochemistry in Non-Small Cell Lung Carcinoma Fine-Needle Aspiration Biopsy

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Key Words

Endobronchial ultrasound · Fine-needle aspiration · Non-small cell carcinoma · p40 immunohistochemistry · Squamous cell carcinoma · Lung cancer

FNA biopsy for pulmonary NSCCA, p40 is a useful IHC marker for neoplastic subclassification, with better specificity in comparison to p63.

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Abstract

Objective: Specific subclassification of pulmonary non-small cell carcinoma (NSCCA) is clinically necessary, and the aim of this study is to examine the utilization of p40 (Δ Np63) in fine-needle aspiration (FNA) biopsy for lung NSCCA. **Study Design:** Database files of the Washington University Medical Center were searched. Patients who underwent endobronchial ultrasound and CT FNA of a primary lung neoplasia were selected and immunohistochemistry (IHC) was performed. A panel of markers was utilized, including p40, p63, cytokeratin (CK) 5/6, thyroid transcription factor, and napsin. **Results:** One hundred patients were identified and comprised 38 squamous cell carcinomas (SCCA), 46 adenocarcinomas (AdCA), and 16 NSCCA. For SCCA, p40 was positive in 34/38 cases (89%) and negative in 4/38 cases (11%); p63 was positive in 33/38 cases (87%) and negative in 5/38 cases (13%); CK5/6 was positive in 38/38 cases. For AdCA cases, p40 was negative, p63 was positive in 2 cases (5%) and CK5/6 was negative in 43/46 cases (92%). **Conclusion:** For NSCCA, p40 had 89% sensitivity and 100% specificity compared to p63 with 86% sensitivity and 96% specificity and CK5/6 with 100% sensitivity and 96% specificity. In the evaluation of

Introduction

With the advent of personalized therapy for non-small cell carcinoma (NSCCA) of the lung, providing a specific neoplastic subtype is necessary in fine-needle aspiration (FNA) and small-caliber biopsies of the lung. Within the NSCCA categorization, this is primarily focused on distinguishing between squamous cell carcinoma (SCCA) and adenocarcinoma (AdCA). The importance is due to the different therapeutic and treatment options which differ between the two. AdCA will be subject to further testing to determine if the use of tyrosine-kinase inhibitors (TKI) are appropriate. SCCA do not benefit from the TKI and do not need to undergo further molecular tests to determine eligibility. For those cases that fall outside of the SCCA category [including AdCA and NSCCA not otherwise specified (NOS)], the additional molecular testing in common practice is currently EGFR and Alk-1. These are performed to identify those patients who will most likely benefit from TKI. TKI, when used in the appropriate setting, have shown considerable survival benefits in patients [1].

Endobronchial ultrasound (EBUS)-guided FNA biopsy is currently the most common and predominant technique utilized for obtaining diagnostic material. CT FNA biopsy also continues to be used. EBUS and CT FNA biopsies have the ability to obtain direct aspirate smears and a cell block preparation. This technique is minimally invasive and frequently diagnostic; however, the relative amount of cellular material collected is modest in comparison to excision of a neoplasm. Since many patients have unresectable tumors at the time of presentation, the EBUS and CT FNA procedure is used for primary diagnosis and to collect sufficient material to guide further therapeutic intervention. The pathologist must use the direct smears and cell block to provide a specific categorization when obtained by EBUS and CT FNA biopsy.

In some instances the cytomorphological features alone will provide a clear and specific subtype classification. However, there are other instances where the NSCCA morphologic appearance is not definitive or clear. In these instances, the use of immunohistochemistry (IHC) has been shown to help in providing a specific diagnostic category [2, 3]. A variety of monoclonal antibodies applied by IHC have been utilized. Different algorithms and antibody mixtures are utilized and advocated. p40 (Δ Np63) is a more recently utilized monoclonal antibody for IHC described in the use of identifying pulmonary SCCA. It has been reported to be more specific for the identification of squamous differentiation [4].

To our knowledge, a side-by-side comparison of p40, p63, and cytokeratin (CK) 5/6 in FNA biopsy cases has not been described. The aim of this study is to examine the use of p40 IHC in cell blocks for primary lung NSCCA sampled by EBUS and CT FNA biopsy.

Materials and Methods

Patients who underwent evaluation for a lung mass and adenopathy were consecutively selected. Those patients with a presumed primary lung malignancy which was diagnosed as NSCCA with a cell block containing adequate material for evaluation were included. Patients with small cell neuroendocrine carcinoma, metastatic neoplasia, and inadequate cell blocks were excluded. IHC was performed on the cell blocks using antibodies for p40 (Δ Np63), p63, and CK5/6, in addition to napsin and thyroid transcription factor (TTF).

Data was collected from the FNA reports and recorded. Direct aspirate smears were both air-dried and alcohol fixed. Alcohol-fixed smears were stained using a Papanicolaou method and air-dried smears were stained via a modified Wright-Giemsa method.

Cell blocks were prepared from needle rinses including various quantities of dedicated aspirate samples collected in RPMI preservative, processed by a thrombin cell block method, fixed in formalin, and paraffin embedded for histologic sectioning and hematoxylin-eosin (HE) staining.

IHC was performed on the selected case cohort. This was performed on representative 4- μ m sections cut from formalin-fixed, paraffin-embedded cell blocks using a commercially available monoclonal antibody to p40 (Δ Np63) (monoclonal; 1:1 dilution), napsin (monoclonal; 1:1 dilution), TTF-1 (monoclonal; 1:1 dilution), p63 (monoclonal; 1:1 dilution), and CK5/6 (monoclonal; 1:1 dilution) on a Ventana Benchmark LT automated immunostainer (Ventana Medical Systems, Inc., Tucson, Ariz., USA) according to standard protocols. Detection involved Ventana's ultraView Universal DAB Detection Kit that utilizes a cocktail of enzyme-labeled secondary antibodies that locate the bound primary antibody. The complex was then visualized with hydrogen peroxide substrate and a 3,3'-diaminobenzidine tetrahydrochloride (DAB) chromogen. No biotin was involved. Antigen retrieval, standard on the machine, utilized Ventana CC1, EDTA-Tris, pH 8.0, solutions. Standard, appropriate histologic tissue was used as the positive control and negative controls for each run. A positive interpretation was judged to be at least 5% of the tumor cells showing at least 2–3 plus intensity staining. A negative interpretation was judged to be an absence of labeling. An equivocal interpretation was judged to be between 1 and 5% of tumor cells or cells of indeterminate morphologic origin showing 1–3 plus intensity staining. This is based on past experience and was utilized as a group for general consistency in reporting. The study was approved by the Institutional Review Board.

Results

One hundred patients were identified. Eighty-one patients underwent EBUS FNA biopsies and 19 CT-guided FNA biopsies. There were 45 men and 55 women. Sixty-two lung lesions and 38 intrathoracic lymph nodes were sampled. CT FNA biopsies were performed exclusively on lung lesions, while the EBUS FNA cases were mixed (43 cases of lung lesions and 38 cases of intrathoracic lymph nodes). p40, p63, and CK5/6 were performed on cell blocks from each case. After cytomorphological evaluation and IHC, the final NSCCA subtypes included 38 SCCA, 46 AdCA, and 16 NSCCA NOS (table 1). Metastatic neoplasms and small cell neuroendocrine carcinomas were excluded by study design.

p40 was utilized in the evaluation of NSCCA. Of the 38 cases subtyped as SCCA, 34 were positive (89%) and 4 were negative (11%) for p40 in the cell block. In comparison, p63 was positive in 33 cases (87%) and negative in 5 (13%). All 38 cases were positive for CK5/6 (fig. 1). For the SCCA subtype, none had positive staining with nap-

Fig. 1. Composite images from one case demonstrating the various staining patterns of a primary pulmonary SCCA in an EBUS FNA. **a** Intermediate sized cell group with abundant eosinophilic cytoplasm without clear keratinization. HE stain, cell block. $\times 40$. **b** p40 shows distinct nuclear labeling of most nuclei within the group. p40 IHC, cell block. $\times 60$. **c** p63 shows distinct nuclear labeling of most nuclei within the group. p63 IHC, cell block. $\times 60$. **d** CK5/6 shows strong cytoplasmic staining with negative images of the nuclei. CK5/6 IHC, cell block. $\times 60$.

sin or TTF; however, there was 1 TTF categorized as equivocal and 4 napsin cases were categorized as equivocal (table 2).

For the 46 cases of AdCA, p40 was negative in all cases (100%). In comparison, p63 was positive in 2 cases (5%), equivocal in 1 (2%), and negative in 43 (93%). CK5/6 was negative in 43 cases (93%) and equivocal in 3 (7%). None of the AdCA cases had a positive p40 or CK5/6. Napsin and TTF were each positive in 44 cases (96%) (table 3).

Of the 16 cases of NSCCA NOS, p40 was negative in 15 cases (94%) and equivocal in 1 (6%), and no cases were positive. In comparison, p63 was negative in all 16 cases (100%). CK5/6 was negative in 8 cases (50%), positive in 2 (12%), and equivocal in 6 (38%). Napsin was negative in 13 cases (81%), positive in 1 (6%), and equivocal in 2 (13%). TTF was negative in all cases (table 4).

For the identification of SCCA in 100 consecutive NSCCA sampled by EBUS and CT FNA biopsy, p40 demonstrated a sensitivity of 89.4%, a specificity of 100%, a positive predictive value (PPV) of 100%, and a negative predictive value (NPV) of 93.9%. p63 demonstrated a sensitivity of 86.8%, a specificity of 96.7%, a PPV of 94.3%, and an NPV of 92.3%. CK5/6 demonstrated a sensitivity of 100%, a specificity of 96.7%, a PPV of 95.0%, and an NPV of 100% (table 5).

Table 1. Clinical characteristics (total patients = 100)

<i>Clinical features</i>	
Men	45
Women	55
EBUS FNA	81
CT FNA	19
Lung lesions (total)	62
Lung lesions (EBUS)	43
Lung lesions (CT)	19
Lymph nodes (intrathoracic)	38
<i>FNA biopsy diagnostic subtype</i>	
SCCA	38
AdCA	46
NSCCA NOS	16

Values are presented as numbers.

Table 2. SCCA IHC findings (n = 38)

IHC:	Positive	Negative	Equivocal
p40	34 (89)	4 (11)	0
p63	33 (87)	5 (13)	0
CK5/6	38 (100)	0	0
Napsin	0	34 (89)	4 (11)
TTF	0	37 (97)	1 (3)

Values are presented as numbers (%).

Table 3. AdCA IHC findings (n = 46)

IHC:	Positive	Negative	Equivocal
p40	0	46 (100)	0
p63	2 (5)	43 (93)	1 (2)
CK5/6	0	43 (93)	3 (7)
Napsin	44 (96)	1 (2)	1 (2)
TTF	44 (96)	2 (4)	0

Values are presented as numbers (%).

Table 4. NSCCA NOS IHC findings (n = 16)

IHC:	Positive	Negative	Equivocal
p40	0	15 (94)	1 (6)
p63	0	16 (100)	0
CK5/6	2 (12)	8 (50)	6 (38)
Napsin	1 (6)	13 (81)	2 (13)
TTF	0	16 (100)	0

Values are presented as numbers (%).

Table 5. Sensitivity, specificity, PPV, and NPV IHC for NSCCA

IHC marker	Sensitivity	Specificity	PPV	NPV
p40	89.4% (75–96)	100% (94–100)	100% (89–100)	93.9% (85–98)
p63	86.8% (71–95)	96.7% (88–99)	94.3% (80–99)	92.3% (82–97)
CK5/6	100% (90–100)	96.7% (88–99)	95.0% (83–99)	100% (93–100)

Values in parentheses are 95% confidence intervals.

Discussion

In current practice, determining a specific subtype of NSCCA is important because of the clinical implications for the patient. In the age of personalized medicine, some types of NSCCA (AdCA and NSCCA NOS) are further evaluated for specific genetic abnormalities to determine their eligibility for TKI, which has been shown to improve survival in this patient subset. On the other hand, SCCAs do not express the genetic profile or benefit from TKI and are therefore not subject to the additional and unnecessary genetic testing. Many patients present with unresectable primary NSCCA, and small sample biopsies are used to establish the diagnosis and provide material for necessary ancillary studies. In a minimal biopsy technique (including EBUS and CT FNA biopsy), a focused approach to IHC can help conserve tissue for potential subsequent genetic testing. A variety of approaches utilizing a mixture of antibodies by IHC have been reported and advocated [2, 3]. These have centered on subtyping AdCA and SCCA, with different individual and combinations/panels of antibodies advocated. More recently, p40 (Δ Np63) has been described as being specific for SCCA [5]. To our knowledge, the concomitant use of p40 and p63/CK5/6 in FNA biopsy material has not been previously described.

p40 (Δ Np63) is present in squamous/basal-type epithelium and corresponds to isoforms of the p63 gene [6]. p63 consists of two major isoforms, i.e. TAp63 and Δ Np63, which differ in the structure of the N-terminal domain. The TAp63 isoform has homology with p53 involved in the regulation of tumor suppressor genes. Δ Np63 contains an inactive Δ N domain which is believed to counter the activity of p53 [7]. The p63 antibody that is generally used in clinical pathology laboratories is directed against both the TAp63 and Δ Np63 isoforms, while the p40 antibody is directed against only the Δ Np63 isoform (and not TAp63).

More recently, reports have described the use of p40 for the identification of SCCA of the lung as distinct from lung AdCA and have shown that it can be specific for SCCA of the lung [4]. Nobre et al. [5], in a review article, discuss the physiologic role of p63 isoforms and their use as diagnostic markers in lung SCCA, and they recommend the use of p40 in discriminating SCCA and AdCA. p63, the more commonly used antibody, has shown good sensitivity for SCCA [2, 8–12], yet it has been shown to have reactivity in non-squamous cell carcinoma, including large cell lymphoma and lung AdCA [9, 13–16].

p40 provides a nuclear staining pattern which is similar in morphologic appearance to p63, in contradistinction to the cytoplasmic decoration seen with CK5/6. In

this series, p40 was interpreted as positive in 34/38 cases of SCCA. p63 was interpreted as positive in 33/38 cases of SCCA. All negative p40 cases were also negative for p63. There was 1 case with p40 positivity and p63 negativity. All of the negative p40/p63 cases had positive CK5/6 staining. In AdCA, 2 cases (2/46) were positive for p63, and none were positive for p40 or CK5/6 (0/46). CK5/6 showed equivocal staining in 7% of the cases (3/46). Of those NSCCA which could not be further subtyped and then grouped as NOS, none were positive for p40 (0/16).

When examining the results of p40 in all 3 categories of NSCCA (SCCA, AdCA, and NSCCA NOS), it had a sensitivity of 89.4%, a specificity of 100%, a PPV of 100%, and an NPV of 93.9%. This sensitivity was slightly better than that of p63 (86.8%), which is the result of 1 case where the p40/p63 was discordant. The specificity of p40 was better than that of p63 (100 vs. 96.7%) and the positivity of 2 cases of AdCA with p63 contributed to this difference. Compared to CK5/6, p40 had better specificity (100 vs. 96.7%) and a better PPV (100 vs. 95.0%). CK5/6 showed better sensitivity (100 vs. 89.4%) and a better NPV (100 vs. 93.9%) in comparison to p40. Overall, p40 demonstrated a better specificity and PPV in comparison to p63 and CK5/6. The sensitivity and NPV were better for CK5/6 compared to p40 and p63.

Our series identified the lower specificity that has been described for p63 in comparison to p40, which has primarily been related to a small subset of focal positivity in lung AdCA [4, 9, 11, 14]. The smaller difference noted in FNA could be related to the relative smaller tumor volume present in a cell block or related to the focal nature of labeling for p63 in lung AdCA which would be more likely to be classified as negative in a small sample cell block. Most of the staining in lung AdCA is focal and involves a minority of cells; however, some cases are described as having strong staining of all cells [4].

We found a higher sensitivity for CK5/6 than p40 or p63 in this group of NSCCA. All of our SCCAs expressed strong cytoplasmic reactivity with CK5/6, and 4 cases lacked both p63 and p40 nuclear expression. These were in cell blocks with distinct but minimal neoplastic elements, which contribute to the interpretation of these results. However, CK5/6 was more often reported as equivocal or more difficult to classify in NSCCA NOS subtype cases (38%) and AdCA (7%). This is likely related to the underlying benign elements frequently admixed. It is common to have some degree of benign pulmonary elements present (from needle aspiration which transverses some uninvolved tissue). Cell blocks are a random mixture of intermediate-to-small groups and single cells,

consisting of benign, reactive, and neoplastic elements in variable proportions. While some small 'micro' biopsy fragments can show a histologic pattern, much of the material present is disrupted and lacks histologic context. This mixed, random 'blender' style collection of cellular material can make definitive classification difficult at times and result in an 'equivocal' designation for CK5/6.

While not the focus of the study design, TTF and napsin were included in the results. Our findings mirrored the experience of others which have been previously reported [3]. TTF and napsin show strong avidity for AdCA. One important point to consider is the occasional staining described in AdCA by p63, which was seen in a number of our cases as well but was lacking in the corresponding p40 staining. Johnson et al. [17] have described a dual stain process utilizing TTF and napsin in the diagnosis of AdCA. This is helpful in providing a specific category diagnosis and in helping to preserve tissue in minimal biopsy specimens where additional ancillary testing (EGFR, Alk, etc.) will be required.

In pulmonary NSCCA diagnosed by EBUS and CT FNA biopsy, p40 has a 100% PPV and higher specificity in comparison to p63 for the subtyping of SCCA. Because of the better performance characteristics, p40 should be considered for use instead of p63 in the evaluation of pulmonary FNA biopsy.

Disclosure Statement

The authors have no financial disclosures to report.

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