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Tissue-Preserving Antibody Cocktails to Differentiate Primary Squamous Cell Carcinoma, Adenocarcinoma, and Small Cell Carcinoma of Lung

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● **Context.**—With the availability of cell type–specific therapies, differentiating primary lung squamous cell carcinomas (SCCs) and adenocarcinomas (ACAs) has become important. The limitations of small sample size and the need to conserve tissue for additional molecular studies necessitate the use of sensitive and specific marker panels on a single slide.

Objective.—To distinguish SCC from ACA and small cell carcinoma (SmCC) of lung using 2 novel tissue-conserving cocktails.

Design.—We compared two antibody cocktails, desmoglein 3 + cytokeratin 5/napsin A and p40/thyroid transcription factor 1 (Biocare Medical, Concord, California) in diagnosing SCC and ACA of the lung on tissue microarray, cytology, and surgical specimens. Both lung and nonlung tissue were evaluated on an 1150-core tissue microarray that contained 200 lung cancers. A microarray of 35 SmCCs and 5 small cell SCCs was also evaluated.

Results.—A cocktail of desmoglein 3 + cytokeratin 5/ napsin A provided diagnostic accuracy in lung cancers with a sensitivity and specificity of 100% in SCCs and a sensitivity of 86% and a specificity of 100% in ACAs. A

p40/thyroid transcription factor 1 cocktail showed p40 to have a specificity of 92% and a sensitivity of 93% in SCCs, whereas thyroid transcription factor 1 had a specificity of 100% and a sensitivity of 77% in ACAs. Cell blocks of fine-needle aspiration cytology compared with corresponding surgical (n = 20) specimens displayed similar findings. The p40 was useful in differentiating bladder from prostate carcinoma with 88% sensitivity. Isolated carcinomas from nonlung tissues were desmoglein 3 + cytokeratin 5 positive. Napsin A was positive in 22% of renal tumors as previously observed. Both cocktails were excellent in differentiating SmCCs and small cell SCCs because none of the SmCCs stained with p40.

Conclusions.—Both antibody cocktails are excellent in differentiating primary lung ACA from SCC, as well as excluding SmCC and ACAs from all other sites on small specimens. A cocktail of desmoglein 3 + cytokeratin 5/ napsin A is slightly superior compared with p40/thyroid transcription factor 1 cocktail.

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D espite more sensitive radiographic imaging techniques and specialized therapies, lung cancer is by far the leading cause of cancer-related mortality worldwide.[1–3] Lung cancer remains the second most common malignancy of men and women in the United States. The American Cancer Society estimates that lung cancer will represent 14% of all new cancers and 28% of all cancer deaths in men and women in 2012.[2] There are a number of different

histologic types of lung cancer, with small cell carcinomas (SmCCs) and non–small cell lung carcinomas (NSCLCs) being the most common. Non–small cell lung carcinomas account for approximately 85% of lung carcinomas. The NSCLCs are further classified into adenocarcinoma (ACA) (40%–50%), squamous cell carcinoma (SCC, 30%) and large cell carcinoma (9%), the latter being composed mostly of poorly differentiated ACA and a few uncommon cell types.[3]

The development of targeted therapies has necessitated the precise typing of NSCLCs. Systematic gene expression differences have been noted among different histologic types of lung cancers.[4–11] The genetic heterogeneity has so far not been found to have a predictive association with standard chemotherapeutic efficacy, but may have a potential utility in histologic subclassification.[12,13] In the past, subtyping NSCLCs did not have therapeutic significance. Now, it is more important than ever to differentiate between ACA and SCC because of type-specific therapies that frequently have a lower incidence of adverse effects while maintaining a highest possible therapeutic response. Recently, oncologic studies have demonstrated that ACA may exhibit a sensitizing mutation to epidermal growth

factor receptors–tyrosine kinase inhibitors resulting in enhanced therapeutic response.[11–13] Additionally, a recombinant humanized monoclonal antibody inhibitor of vascular endothelial growth factor, bevacizumab (Avastin; Genentech, Inc, San Francisco, California), which has been found to be effective when used in combination with standard first-line chemotherapy, has been associated with life-threatening hemorrhage in lung SCC, particularly the cavitating variant. The addition of the antifolate agent pemetrexed to conventional chemotherapy provides increased efficacy in nonsquamous carcinomas, but not in SCC.[14] Therefore, histologic differentiation of NSCLCs is vital for improved treatment response and reduces adverse effect.[15]

In most cases, standard hematoxylin-eosin (H&E) evaluation provides sufficient information to classify NSCLC cases. However, accurate diagnosis can be limited in small biopsies or cytology specimens, in poorly differentiated neoplasms, and in cases where there is marked disruption of histologic architecture. A review of 303 primary lung cancer resections showed that 72% were diagnosed by biopsy or cytology, either alone or in conjunction.[16] In addition to limited tissue available for review, the concordance rates among pathologists vary significantly. In 2006, concordance rates among pathologists subtyping NSCLCs on H&E alone were as low as 81%.[17] Furthermore, NSCLCs represent primary lung malignancies with histologically indistinguishable counterparts in other organs. This leads to a potential diagnostic pitfall in both ACA and SCC due to the difficulty in differentiating a primary lung carcinoma from histologically identical lung metastasis with an unidentified primary source. Misdiagnosis in cases of unidentified primary lesions with metastasis to the lung can result in understaging and suboptimal treatment. Thus far, lung specific markers for SCC have been limited, whereas napsin A and thyroid transcription factor 1 (TTF-1) have proven to be useful in the diagnosis of primary lung ACAs.

A diagnosis of SCC is supported by presence of unequivocal cytoplasmic keratinization, squamous pearls, and desmosomes. A mucin stain is a useful adjunct to conventional H&E in diagnosis of ACA to demonstrate glandular differentiation or lepidic growth pattern. Lack of these features may render an accurate classification difficult on H&E stain, and the difficulty may be compounded by the limited tissue on fine-needle biopsies, which are being more frequently used for diagnosis.[17]

The implementation of immunohistochemistry has become a well-supported and accessible tool for the accurate diagnosis and typing of carcinoma, including primary lung carcinomas. Additionally, immunohistochemistry-derived immunophenotypes can allow for organ-specific differentiation of histologically indistinguishable primary and metastatic carcinomas. Given the therapeutic and prognostic information that stems from an accurate histologic typing of lung cancers, a number of immunohistochemical markers have been studied.[18] Most of the studies recommend use of panels of antibodies rather than a single antibody to increase the sensitivity and specificity. The panels have included TTF-1 with carcinoembryonic antigen for ACA and cytokeratin (CK) 5/6 and p63-desmoglein 3 (DG3) for SCC,[18–20] as well as TTF-1 and napsin A for ACA and p63 with CK5/6 for SCC.[21]

Thyroid transcription factor 1 is a 38-kDa homeodomain protein that shows nuclear-specific staining. It regulates gene expression in the thyroid, lungs, and diencephalon during embryogenesis. It is normally expressed in alveolar pneumocytes, Clara cells, ciliated respiratory epithelial cells, and basal cells of the lung.[22] The use of TTF-1 has been well established for differentiation between primary and metastatic ACA of the lung. Although TTF-1 is considered a relatively restricted marker with high sensitivity, the reported sensitivity for lung ACA has been as low as 54%.[23–30]

Napsin A is a newer antibody marker for pulmonary ACAs. It is a functional aspartate proteinase involved in the maturation of prosurfactant protein B in type II pneumocytes, and in the maturation of the biologically active surfactant protein B. This single chain protein is normally expressed in type II pneumocytes, alveolar macrophages, renal tubules, exocrine glands, and pancreatic ducts.[24,31] The role of napsin A in differentiating primary from metastatic ACA of the lung has been previously reported.[21,23,32–34] Although it may occasionally stain nonpulmonary ACAs, it is a highly useful marker in differentiating primary lung ACAs from SCCs.[33–35] Positive immunohistochemical stain shows intense granular cytoplasmic reactivity.[21,31,34,35]

p63 is a member of the p53 family. Located on chromosome 3q27–29, it is involved in the regular growth and development of epithelial tissue.[36] In normal tissues, p63 has been reported to be positive in basal cells of all squamous epithelia, in basal cells of urothelium, and in basal cells of prostate epithelium.[37,38] p63 is detectable in most SCCs of various primary sites, including SCCs of lung, with reported positivity of 80% to 97% in most studies.[39–43] In the lung, however, p63 has been shown to have some overlap in ACAs, with reported positivity in up to 18%.[37–40] To correctly interpret p63, only nuclear staining should be considered as positive.

Also from the p53 family, p40 appears to be a specific marker of squamous cell differentiation related to the nontransactivating isoforms of the p63 gene family of nuclear transcription factors.[44–46] The p63 gene encodes diverse messenger RNA isoforms, which are generated by the activity of 2 different promoters that leads to the accumulation of transactivating p63 isoforms (ΔNp63-p40), which may act as negative dominant agents that lead to the stimulation of cell proliferation, block apoptosis, and allow for unrestrained tumor cell growth.[46] There are very few reports of ΔNp63-p40 expression in lung cancers.[42,44,45]

Cytokeratins are the dominant, intermediate filament proteins of the epithelial cells. Cytokeratins 5 and 6 are related proteins that can be detected in normal cells, including breast myoepithelial cells, prostate basal cells, and the basal layer of the epidermis and salivary glands. Positive immunohistochemical staining displays a membranous staining pattern. Marson et al[25] reported positive staining in 100% of primary lung SCCs, which may be an overestimation in our experience.

Desmogleins are one of the major glycoproteins of the desmosomal structure found in epithelial cells. They are calcium-dependent adhesion molecules that belong to the cadherin superfamily and link to CK filaments via desmoplakins and plakoglobin. Therefore, their expression is also membranous and not cytoplasmic in epithelial cells. Furthermore, DG3 in particular has an important role in cellular adhesion of stratified epithelia such as squamous epithelium and is highly expressed in SCCs of the lung.[47–50] Savci-Heijink et al[20] described a sensitivity of 99% and specificity of 87% for SCCs in primary tumors from different

sites. In primary lung tumors, they described staining in 98% of SCCs, and 99% of non-SCCs were negative.

Recently, Ring et al[51] described a 5-antigen commercial panel to subclassify NSCLC that comprised CK5/6, MUC1, the carcinoembryonic adhesion molecule (CEA-CAM5), TRIM29, and SLC7A5.56. They demonstrated a greater sensitivity when this panel was compared with a panel of TTF-1/p63 (88.6% versus 74.1%); specificity was equal between the 2 methods. Yanagita et al[52] created an immunostaining method using a 4-antibody cocktail of TTF-1, napsin A, p63, and CK14 with high specificity and sensitivity for differentiating ACAs and SCCs of the lung. The aim of this study is to identify a small, accurate, and cost-effective immunohistochemical panel that would enable differentiation of primary lung ACAs from primary lung SCCs on limited material, preferably using 1 or 2 slides.

MATERIALS AND METHODS

Tissue Microarray for NSCLC

Tissue microarrays were made available by the Laboratory of Pathology, Toyama University Hospital (Toyama, Japan). The material consisted an of unstained, formalin-fixed, paraffin-embedded 1150-core tissue microarray of lung and nonlung tissue; nonlung organs included thyroid, breast, stomach, biliary tract, liver, colon, pancreas, kidney, bladder, prostate, ovary, and uterine corpus. The tissue cores were 0.6 mm in diameter and collected from resected specimens with a confirmed H&E diagnosis. The microarray included SCCs (n = 90) and ACAs (n = 93) of the lung, as well as renal cell carcinoma (n = 98) and hepatocellular carcinoma (n = 98). Other sampled organs included neoplastic and nonneoplastic tissue cores.

Cytologic and Surgical Comparison Sets

In order to evaluate staining consistency and reliability in cytologic specimens, we collected unstained sections of paraffin blocks containing cytologic and surgical specimens from 11 patients with resection-proven pulmonary ACA and 11 patients with pulmonary SCC from the pathology archives of a large tertiary care hospital (Department of Pathology, University Hospital, San Antonio, Texas). All cases were resected, fixed, and paraffin embedded between 2002 and 2012, and required positive identification of neoplastic cells in both cytologic and surgical specimens at the time of collection. Additionally, attempts to identify neoplastic cells on cytologic smears retrieved from the original evaluation were performed. Aspirate smears originally stained with Romanowsky stain (Diff-Quik) were decolorized. Attempts to recover positive staining in neoplastic cells with and without antigen retrieval were performed.

Tissue Microarray for SmCC and Small Cell SCC

A second tissue microarray provided by the Department of Pathology at The Methodist Hospital (Houston, Texas) was stained and reviewed. The formalin-fixed, paraffin-embedded tissue block contained 0.6-mm cores from 35 SmCCs and 5 small cell SCCs.

Immunohistochemistry

All immunohistochemical staining was performed at our histology laboratory (The University of Texas Health Science Center at San Antonio Histology Laboratory, San Antonio, Texas) using 2 dual-antibody cocktails for DG3 + CK5/napsin A and p40/TTF-1 (prediluted; napsin A and p40, rabbit polyclonal; DG3 + CK5 and TTF-1, mouse monoclonal; Biocare Medical, Concord, California). The slides were treated for 30 minutes at 100°C and then allowed to cool for 10 minutes. The antigen retrieval step was bypassed in 2 sets of cytologic smears to evaluate staining quality. The slides were incubated with primary antibody for 30 minutes followed by double staining detection with MACH 2 double stain 2 for 30 minutes and developed with diaminobenzidine brown

chromogen for TTF-1 and DG3 + CK5 and warp red for p40 and napsin A.

After staining, the tissue microarray slides were scanned by Spectrum Plus (Aperio Technologies, Inc, Vista, California) at high-power resolution (×400) to a dedicated server. Granular cytoplasmic staining in more than 1% of tumor cells was scored positive for DG3 + CK5 (brown) and napsin A (red). A positive nuclear staining in more than 1% of tumor cells was scored positive for p40 (red) and TTF-1 (brown); otherwise the tissue was scored negative. All results were evaluated against a negative control from the same tissue microarray. Each cocktail served as a quality control for the other. The comparison cases were evaluated using high-power resolution and were scored as positive if greater than 1% of tumor cells displayed positivity for the evoked stain. Anticipated internal controls and aberrant staining patterns including edge artifact and necrotic or apoptotic debris were not included in the scoring. All stained slides were compared against a negative control from the same tissue block.

RESULTS

We compared the staining profiles of 2 antibody cocktails, DG3 + CK5/napsin A and p40/TTF-1, in diagnosing NSCLC on tissue microarray, surgical, and cytologic specimens (Figures 1 and 2). Staining of the Toyoma tissue microarray yielded 183 cases of NSCLC, including 93 ACAs and 90 SCCs. All 4 stains displayed high specificity for their designated cell markers (Table 1); SCCs were stained by p40 and DG3 + CK5 whereas ACAs were stained by TTF-1 and napsin A. Desmoglein 3 + CK5 showed 100% sensitivity for SCCs, whereas p40 was comparatively less sensitive at 93% for SCCs. Napsin A displayed superior staining for ACAs, with a sensitivity of 86% compared with 77% by TTF-1. Overall, the DG3 + CK5/napsin A cocktail appeared to be the superior stain on review of lung tissues in tissue microarray (Figure 3). Both staining panels provided excellent diagnostic accuracy for NSCLCs with significant parallel concordance in all cases. Only one case marked with both ACA and SCC markers on both cocktails; it was reclassified as an adenosquamous cell carcinoma because the morphology supported this as well in retrospect.

Staining of nonlung tumor tissue revealed significant sensitivity in both staining panels (Table 2). Thyroid transcription factor 1 remained specific to ACA of the lung and thyroidal tissues and displayed negative staining of other tissues. Only one case of bladder ACA aberrantly expressed TTF-1. As expected, a majority of thyroid neoplasms (84.2%) showed nuclear TTF-1 staining. Napsin A appeared more sensitive than TTF-1 at detecting lung ACA but showed positivity in 22% of renal cell carcinoma, previously shown to be papillary and clear cell variants.[52] Overall, TTF-1 and napsin A displayed greater than 92% specificity for lung ACA throughout all organs sampled with napsin A alone, reaching a specificity of greater than 99% when renal tissues were eliminated. Dual expression of napsin A and TTF-1 was specific for pulmonary tissue except in some cases of thyroid carcinoma.

The staining pattern for p40 showed high affinity for lung SCC, but also showed positivity in 88% of urothelium cores (42 of 48), which is similar to previously reported findings in p63 evaluation.[36] Additionally, p40 showed consistent strong aberrant apical cytoplasmic staining of colonic mucosa, similar to the staining pattern seen with some other keratins. The DG3 + CK5 cocktail maintained high sensitivity for lung SCC, with rare staining of ACAs from nonlung tissues including ACAs from the pancreas, uterine endometrium, breast, and biliary tract. A single pancreatic
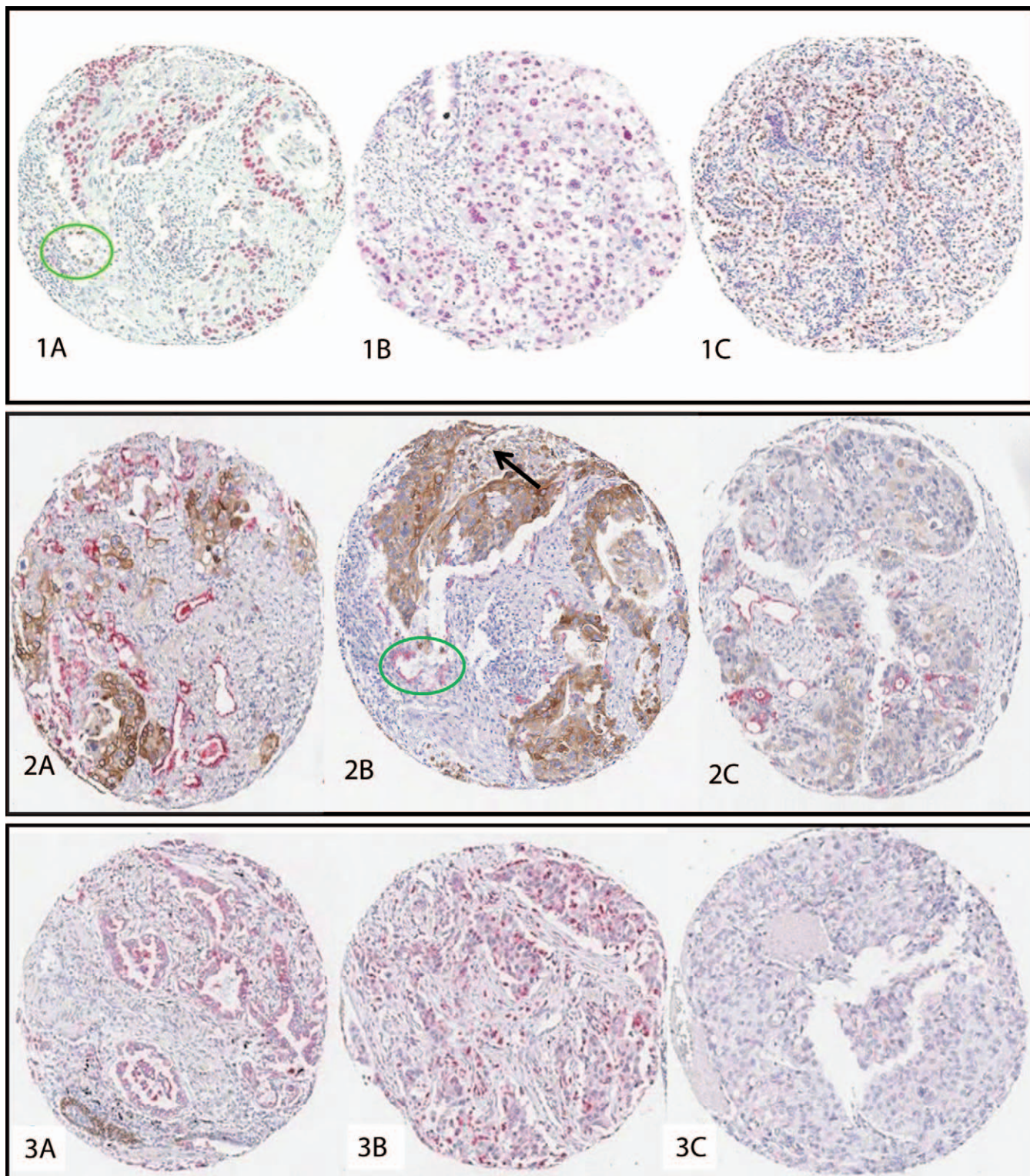
Figure 1. *Thyroid transcription factor 1/p40 cocktail immunostain; p40 (red) with crisp nuclear staining of well-differentiated (A) and poorly differentiated (B) squamous cell carcinoma. Thyroid transcription factor 1 (brown) displays strong nuclear staining in an adenocarcinoma (C). Note background type II pneumocyte nuclear staining for thyroid transcription factor 1 in A (circle) (original magnifications ×40).*

Figure 2. *Desmoglein 3 + cytokeratin 5/napsin A cocktail immunostain. A, Napsin A (red) with staining of type II pneumocytes adjacent to desmoglein 3 + cytokeratin 5 (brown) stained squamous cell carcinoma. B, Necrotic areas (arrow) in squamous cell carcinoma staining with desmoglein 3 + cytokeratin 5 and type II pneumocytes (circle) staining for napsin A. C, Adenosquamous carcinoma with dual napsin A and desmoglein 3 staining (original magnifications ×40).*

Figure 3. *Desmoglein 3 + cytokeratin 5/napsin A cocktail immunostain; napsin A (red) displaying variable staining intensity in well differentiated (A) and moderately differentiated (B) and weak staining in poorly differentiated (C) adenocarcinoma. Note the desmoglein 3 + cytokeratin 5 (brown) staining of metaplastic squamous epithelium (original magnifications ×40).*

Antibody Cocktails to Differentiate Lung Carcinoma—Brown et al

Table 1. Staining of Non–Small Cell Lung Carcinoma on Tissue Microarray[a]

	ACA Markers		SCC Markers	
	TTF-1	Napsin A	p40	DG3 + CK5
ACA (n = 93)	72	80	7	0
SCC (p40, n = 89; DG3 + CK5, n = 90)	0	0	83	90
Sensitivity, %	77.4	86.0	93.3	100
Specificity, %	100	100	92.4	100
PPV, %	100	100	92.2	100
NPV, %	80.9	87.3	93.5	100

Abbreviations: ACA, adenocarcinoma of the lung; DG3 + CK5, desmoglein 3 and cytokeratin 5; NPV, negative predictive value; PPV, positive predictive value; SCC, squamous cell carcinoma of the lung; TTF-1, thyroid transcription factor 1.

[a] One case was determined to be adenosquamous carcinoma with positivity for all 4 stains.

Table 2. Staining of Nonlung Tissues on Toyoma 1150-Core Tissue Microarray

		% Positive			
Tissue	No. of Samples	TTF-1	Napsin A	P40	DG3 + CK5
Breast	95	0	0	1.1	3.2
Kidney (RCC)	98	0	22.4	0	0
Biliary tract	91	0	0	3.3	3.3
Thyroid	95	84.2	0	0	0
Liver (HCC)	98	0	0	0	0
Colon (CACA)	95	0	0	0	0
Stomach (SACA)	92	0	0	0	0
Pancreas (PACA)	48	0	0	2.1	2.1
Urothelial carcinoma	48	2.1	0	88.0	27.1
Ovary (epithelial)	47	0	0	0	0
Uterus (endometrial)	48	0	0	2.1	2.1
Prostate (PRACA)	44	0	0	2.3	0

Abbreviations: CACA, colonic adenocarcinoma; DG3 + CK5, desmoglein 3 and cytokeratin 5; HCC, hepatocellular carcinoma; PACA, pancreatic adenocarcinoma; PRACA, prostatic adenocarcinoma 1; RCC, renal cell carcinoma; SACA, stomach adenocarcinoma; TTF-1, thyroid transcription factor.

malignancy displayed p40 and DG3 + CK5 positivity, which may represent an adenosquamous or squamous carcinoma. Three cases of ductal breast carcinoma were positive for DG3 + CK5, one of which was also p40 positive, which may possibly represent a metaplastic carcinoma or a basal phenotype. Three cores from the biliary tract were DG3 + CK5 positive and 3 separate cases were p40 positive; no cases had dual staining.

Desmoglein 3 + CK5 and p40 were only rarely positive in uterine endometrial ACA (p40 and DG3 + CK5 in 1 of 48) and prostate (p40 in 1 of 44). All ACAs and tumors from the ovary, liver, colon, and stomach were negative for DG3 + CK5 and p40.

Comparison sets of cytologic and surgical specimens from 11 patients with resection-proven ACA were collected. One case was entirely removed (cytology and surgical specimens) from the study after it was found to be a metastatic pulmonary nodule in a patient with colonic ACA with negative staining. The paraffin block for one cytology specimen was exhausted and no tissue was available for staining. Within the 9 recovered cytologic cases, 100% displayed staining for both TTF-1 and napsin A. All of the cytology cases of pulmonary ACA were negative for DG3 + CK5 and p40. All corresponding surgical cases (n = 10) were recovered, with 100% of cases expressing positivity for napsin A and TTF-1 (Table 3). Only 1 of the surgical cases showed aberrant DG3 + CK5 and p40 positivity within tumoral glands. Overall, DG3 + CK5 displayed staining in basal cells, which served as a reliable internal control. Additionally, TTF-1 was observed to have a high nuclear affinity, allowing for greater confidence when confirming positivity in cytologic specimens compared with napsin A, because the latter also stained alveolar macrophages. Though incidental, the full panel negativity on both cocktails in a case of metastatic colonic ACA reinforced the lung-based specificity of this staining panel.

Comparison sets of cytologic and surgical specimens from 11 patients with resection-proven SCC were collected and evaluated using the 2 cocktails (Table 4). The cytologic cases (n = 11) displayed 64% (7 of 11) positivity for p40 (Figure 4) and 100% positivity for DG3 + CK5 (Figure 5). Two of the 4 SCC cases found to be negative for p40 were described as necrotic in the final diagnosis comment; the remaining 2 cases that were negative were classified as poorly differentiated SCC. All of the cytologic specimens of SCC were negative for TTF-1 and napsin A. Napsin A maintained staining in alveolar macrophages, which served as a reliable internal control. The surgical cases (n = 11) displayed 91% (10 of 11) positivity for p40 and 100% positivity for DG3 +

Table 3. Matched Cytology and Surgical Cases of Adenocarcinoma

	Cytology				Surgical Biopsy				
Patient No.	DG3 + CK5	p40	TTF-1	Napsin A	DG3 + CK5	p40	TTF-1	Napsin A	Diagnosis
1	−	−	+	+	−	−	+	+	ACA, PD
2	−	−	+	+	−	−	+	+	ACA, PD
3	−	−	+	+	−	−	+	+	ACA, PD
4	Lost	Lost	Lost	Lost	−	−	+	+	ACA, MD
5	−	−	+	+	−	−	+	+	ACA, PD
6	−	−	+	+	−	−	+	+	ACA, PD
7	−	−	+	+	−	−	+	+	ACA, PD
8	−	−	+	+	−	−	+	+	ACA, MD
9	−	−	+	+	1+	1+	+	+	ACA, MD
10	−	−	+	+	−	−	+	+	ACA, PD
11	−	−	−	−	−	−	−	−	Colonic ACA
Total	**0**	**0**	**9**	**9**	**1**	**1**	**10**	**10**	
%	0	0	90	90	10	10	91	91	

Abbreviations: ACA, adenocarcinoma; DG3 + CK5, desmoglein 3 and cytokeratin 5; Lost, tissue lost on subsequent slides; MD, moderately differentiated; PD, poorly differentiated; TTF-1, thyroid transcription factor 1; 1+, weak, focal positivity; +, positive; −, negative.

Table 4. Matched Cytology and Surgical Cases of Squamous Cell Carcinoma									
	Cytology				Surgical Biopsy				
Patient No.	DG3 + CK5	p40	TTF-1	Napsin A	DG3	p40	TTF-1	Napsin A	Diagnosis
1	+	−	−	−	+	+	−	−	SCC, necrotic
2	+	+	−	−	+	+	−	−	SCC, MD
3	+	+	−	−	+	+	−	−	SCC, necrotic
4	+	−	−	−	+	+	−	−	SCC, PD
5	+	+	−	−	+	+	−	−	SCC, PD
6	+	+	−	−	+	−	−	−	SCC, necrotic
7	+	+	−	−	+	+	−	−	SCC, PD
8	+	−	−	−	+	+	−	−	SCC, PD
9	+	+	−	−	+	+	−	−	SCC, MD
10	+	+	−	−	+	+	−	−	SCC, MD
11	+	−	−	−	+	+	−	−	SCC, necrotic
No.	11	7	0	0	11	10	0	0	
%	100	64	0	0	100	91	0	0	

Abbreviations: DG3 + CK5, desmoglein 3 and cytokeratin 5; MD, moderately differentiated; PD, poorly differentiated; SCC, squamous cell carcinoma; TTF-1, thyroid transcription factor 1; +, positive; −, negative.

CK5. One surgical case was negative for p40 and was described as necrotic in the final diagnosis comment. The p40 stain appears to be highly specific and results in a crisp, strong nuclear stain that is clearly positive. Alternatively, p40 appears to lack sensitivity in cytologic specimens and specimens with nonoptimal factors like tissue necrosis or poorly differentiated histologic grade. The DG3 + CK5 stain showed excellent sensitivity and specificity in both cytologic and surgical specimens, including poorly differentiated and necrotic SCC.

In the SmCC microarray, all cases (n = 35) were identified by a strong positive staining for TTF-1 and negative staining for napsin A, DG3 + CK5, and P40, providing a negative predictive value of 100% for the DG3 + CK5/napsin A cocktail. Additionally, 5 small cell SCC cases included on the same microarray were positive for p40 and DG3 + CK5. Only 5 cases on this microarray were negative for all markers and were determined to be large cell carcinomas.

COMMENT

Immunohistochemistry is a powerful adjunct to conventional morphology and can be well applied to non–small cell and small cell carcinoma lung carcinomas where tissue is limited and targeted therapies impose a strain on pathologists to establish a reliable classification. The use of immunohistochemistry in subtyping NSCLCs has increased since the advent of targeted lung therapies. A review by Ocque et al[53] found that the frequency of immunohistochemical studies in typing NSCLCs has risen to 89% for SCCs and 85.9% for ACAs between 2005 and 2010 compared with 11% and 14.1% from 2000 to 2004, respectively. The use of immunohistochemistry in NSCLCs clearly increases interobserver diagnostic accuracy, and various antibodies are currently being used to aid in differentiating SCC from ACA of lung.[52] The need to conserve diagnostic tissue for molecular testing further necessitates the discovery of an accurate, reliable, and cost-effective set of immunohistochemical markers.

In comparing the 2 antibody panels, a cocktail of DG3 + CK5/napsin A provides excellent diagnostic accuracy in lung cancers with a sensitivity and specificity of 100% for SCCs and a specificity of 100% and a sensitivity of 86% in ACAs on lung tissue microarray. In comparison, the p40/TTF-1 panel displays an overall specificity of 92% and sensitivity of 93% for SCCs and specificity of 100% and sensitivity of 77%
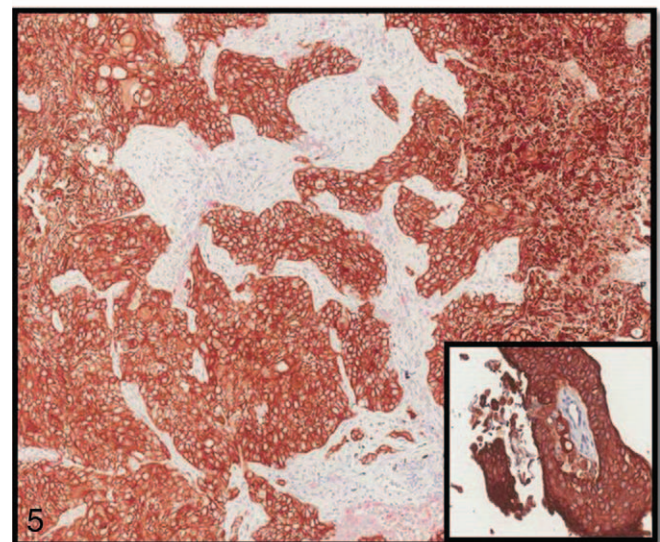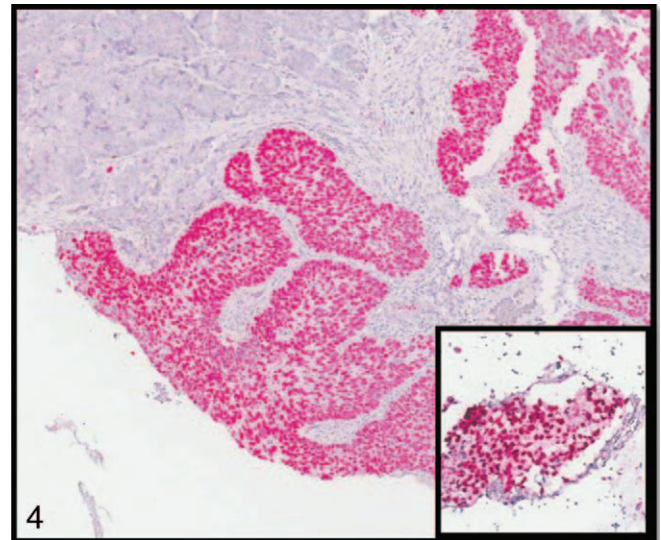

Figure 4. Thyroid transcription factor 1/p40 cocktail immunostain; nuclear p40 staining of squamous cell carcinoma in surgical excision and fine-needle aspirate (inset) specimens (original magnifications ×200 and ×400 [inset]).

Figure 5. Desmoglein 3 + cytokeratin 5/napsin A cocktail immunostain; cytoplasmic desmoglein 3 + cytokeratin 5 staining of squamous cell carcinoma in surgical excision and fine-needle aspirate (inset) specimens (original magnifications ×200 and ×400 [inset]).

for ACAs on lung tissue in microarray. In a review of the literature, our data are similar with the previously reported studies using these antibodies individually. Mukhopadhyay and Katzenstein[54] evaluated the utility of napsin A, TTF-1, CK5/6, and p63 in subtyping poorly differentiated NSCLC. Assessment using the immunohistochemistry panel allowed for correct subtyping on 77% of cases (30 of 39) that initially could not be classified by morphology alone at the time of biopsy.[54] As previously referenced, Yanagita et al[52] created an immunostaining method using a 4-antibody cocktail of TTF-1, napsin A, p63, and CK14 for subtyping NSCLCs. The use of 2 markers each for ACA and SCC provided confident subtyping in 97.4% of ACAs (n = 39) and 100% of SCCs (n = 25) using only 2 slides.[51] The DG3 + CK5/napsin A cocktail, unlike the YANA-5 cocktail used in the Yanagita et al[52] study, simultaneously allows for elimination of SmCC.

The DG3 + CK5/napsin A cocktail had 100% negative predictive value in 35 cases of SmCCs on tissue microarray with both cocktails. The stains were also able to distinguish 5 cases of small cell SCC from SmCCs included on the same tissue microarray. The TTF-1/p40 cocktail displayed strong TTF-1 staining in SmCC and strong p40 staining in 5 included small SCC cases. These findings provide additional confidence in distinguishing primary lung carcinoma on limited tissue specimens.

Within nonlung tissues, the immunohistochemistry panels retained excellent specificity with very rare examples of aberrant staining. The use of p40 to differentiate bladder from prostate carcinoma in cases of local invasion appears to be a viable alternative use of the cocktail. On tissue microarray, 88% of bladder (urothelial) carcinoma displayed positivity for p40, whereas only one case of prostatic ACA displayed staining for p40. These findings are similar to those of previous studies assessing p63 immunostaining.[42] Additionally, p40 showed consistent aberrant cytoplasmic staining in the apical pole of colonic ACA, which may be a unique distinguishing feature for colon cancer that can be exploited. The use of napsin A should be limited in cases where metastatic renal cell carcinoma is in the differential diagnosis. Renal cell carcinoma on tissue microarray displayed 22% positivity for napsin A. Napsin A has been shown to have significant staining in renal cell carcinomas, particularly in the papillary subtype.[35] As expected, TTF-1 showed strong nuclear positivity in 84% of thyroid tissue.

Both cocktails are very valuable on cytology material in accurately subclassifying SCC versus ACA versus SmCC. The p40/TTF1 combination is favored in this setting because napsin A does stain alveolar macrophages, whereas p40/TTF-1 displays crisp nuclear staining.

The results of this study demonstrate a cocktail of DG3 + CK5/napsin A and TTF-1/p40 to be an excellent and cost-effective choice when faced with differentiating primary SCCs versus ACAs versus SmCC of lung. The choice of napsin A over TTF-1 is favored because of TTF-1 expression in thyroid tumors as well as neuroendocrine tumors of the lung.[54–59] Desmoglein 3 + CK5 appears to be a very sensitive and specific marker with higher sensitivity and specificity than any antibody panels used previously for SCC.[20] In the evaluation of both tissue microarrays and comparison case sets, DG3 + CK5/napsin A appears to be the superior staining panel based on the observed sensitivity, specificity, and reliability. Use of both staining panels, DG3 + CK5/napsin A and p40/TTF-1, appears to increase diagnostic accuracy and should be considered for secondary evaluation in difficult cases with questionable DG3 + CK5/napsin A

staining. A rare tumor gland on biopsy material may express DG + CK5 aberrantly and should not pose a problem because the gland is readily recognizable. Five of the 183 tumors remained truly unclassifiable, which is 2.7% and close to or lower than observations made by other authors.[60]

CONCLUSION

To our knowledge, this is the first study comparing the diagnostic efficacy of TTF-1, napsin A, p40, and DG3 + CK5 in differentiating primary SCCs and ACAs of lung. Use of the 2 marker cocktails, DG3 + CK5/napsin A and p40/TTF-1, provided excellent sensitivity and specificity for subtyping NSCLCs against SmCC of the lung and can reliably distinguish lung ACA from SCC on single slides. The cocktails showed dependable sensitivity for primary lung tumors with rare aberrant positivity in nonlung tissues, outside of well-documented staining patterns.[61] The results of the DG3 + CK5 and napsin A cocktail appear superior to those of TTF-1 and p40, making DG3 + CK5/napsin A the antibody cocktail of choice for distinguishing SCC from ACA. Both cocktails are needed when distinguishing SCC versus ACA versus SmCC.

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