A 6-Antibody Panel for the Classification of Lung Adenocarcinoma Versus Squamous Cell Carcinoma

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Abstract: Non-small cell lung cancer can be classified into several histologic subtypes, most commonly lung adenocarcinoma (LADC) or squamous cell carcinoma (SqCC). With the introduction of targeted therapies that can result in dramatically different outcomes based on subtype, the importance of accurate classification has been amplified. Six antibodies (Napsin A, Desmoglein 3, TTF-1, CK5, p63, and tripartite motif-containing 29) were selected for evaluation on cases of LADC of lung SqCC. Guided by the sensitivities and specificities determined for individual antibodies, a protocol was developed using a sequential series of 2-antibody cocktails that resulted in the classification of 95% of cases with 100% specificity. Importantly, the initial step in this method, a napsin A + Desmoglein 3 antibody cocktail classified >85% of cases, resulting in <15% of cases requiring further evaluation beyond a single test. Two new antibodies specifically developed and optimized for the diagnosis of LADC and lung SqCC, a rabbit polyclonal Napsin A and a mouse monoclonal Desmoglein 3 [BC11], were key elements of the antibody panel. Most importantly, the described protocol uses routine interpretation methods and an uncomplicated algorithm for classification. Given the increased difficulty of diagnosing poorly differentiated tumors, the ability of this 6-antibody panel to classify 96% and 87% of moderately and poorly differentiated cases, respectively, is of particular value, especially when limited tissue for molecular testing is an issue.

Key Words: lung adenocarcinoma, lung squamous cell carcinoma, Napsin A, Desmoglein 3, TRIM29

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Lung cancer is the leading cause of cancer deaths among both men and women. More people die of lung cancer than of colon, breast, and prostate cancers combined.1 Non-small cell lung carcinoma (NSCLC) comprises approximately 80% of lung cancers and can be classified into several histologic types, most commonly adenocarcinoma or squamous cell carcinoma (SqCC). Classification of lung carcinomas into histologic types is typically carried out by morphologic examination using hematoxylin and eosin or immunohistochemistry, and in some cases, mucin stains.2–5 However, an accurate classification can be difficult with poorly differentiated lung carcinoma. Diagnosis can be further complicated by the use of core needle biopsies, which provide limited amounts of tissue for both immunohistochemistry and molecular testing, and may include crush artifacts. Many lung tumors are unresectable at diagnosis, thus requiring accurate chemotherapy and/or targeted radioteherapy.

Although the majority of lung cancers (particularly grades I and II) can be diagnosed with only hematoxylin and eosin staining, diagnostic parameters have changed and a more detailed approach to diagnosis has emerged. Furthermore, the availability of targeted therapies has created a need for accurate subtyping of NSCLC. The current Food and Drug Administration-approved standard of treatment for NSCLC is carboplatin/taxol/avastin; however, patients with lung SqCC should not receive avastin due to a 30% mortality rate by fatal pulmonary hemorrhage.5,7 Historically, the antibodies TTF-1 and p63 have often been used to differentiate primary adenocarcinoma from SqCC of the lung.8–13 Recently, a panel of TTF-1, p63, Napsin A, and CK5/6 was used to classify 77% of poorly differentiated cases of NSCLC; however, 23% of the cases remained unclassified.2 Similarly, a 5-antibody panel that included CK5/6, tripartite motif-containing 29 (TRIM29), LAT-1, CEACAM5, and MUC1 used a weighted mathematical formula to classify 85% of lung adenocarcinoma (LADC) cases and 88% of lung SqCC cases, respectively, while leaving 12.8% of the cases unclassified.5 Two new antibodies, TRIM29 and Desmoglein 3 (DSG3), have also emerged as potential markers for SqCC lung cancer; however, only limited studies of these antibodies have been reported.5,14,15 With the more stringent requirements for histologic classification of lung cancers, the need for a panel of antibodies that easily and definitively differentiates LADC from lung SqCC is of utmost importance.

In a pilot study, 15 antibodies were evaluated for their sensitivity and specificity for LADC and lung SqCC,
using an immunohistochemical (IHC) method (Supplementary Table 1; Supplemental Digital Content-1 http://links.lww.com/AIMM/A17). As part of this study, a Napsin A rabbit polyclonal antibody and DSG3 [clone BC11] mouse monoclonal antibody were generated and optimized with special emphasis for their utility for lung cancer diagnosis. On the basis of sensitivity and specificity determined in the pilot study, 6 antibodies (TTF-1, Napsin A, p63, TRIM29, DSG3, and CK5) were selected for further evaluation on 210 various lung cancer cases. Lung cancer classification was divided by phenotype (adenocarcinoma vs. SqCC), and in each group that had previously been diagnosed as well, moderately, or poorly differentiated grades I, II, and III were assigned, respectively. Analysis of the staining percentages for each antibody facilitated the development of an algorithm that can be used to classify >93% of cases as LADC or SqCC.

**MATERIALS AND METHODS**

Formalin-fixed, paraffin-embedded tissue microarrays (TMAs) of various lung cancer cases were processed in the usual manner for IHC analysis. TMA core tissues (US Biomax) were randomly selected from previously diagnosed material and patient data included age, tumor grade, and staging. The cases evaluated included 156 men with an average age of 60 years and 54 women with an average age of 58 years. All tissue sections were retrieved in a modified citrate buffer (DIVA, Biocare Medical) in a pressure cooker (Decloaking Chamber, Biocare Medical) at 125 °C. Staining of TTF-1 [8G7G3/1], Napsin A (P), p63 [4A4], TRIM29 (P), DSG3 [BC11], and CK5 [EP1601Y] (Biocare Medical) was optimized with custom diluents, based on sensitivity and specificity (Supplementary Table 2; Supplemental Digital Content-2 http://links.lww.com/AIMM/A18). Antibodies and/or antibody cocktails were evaluated on 95 cases of lung SqCC and 115 cases of LADC. Detection was achieved using a micropolymer detection system conjugated to horseradish peroxidase or alkaline phosphatase (MACH 2, Biocare Medical) and visualized with DAB and/or Fast Red, respectively.

**Scoring Method for Interpretation**

Scoring and interpretation methods were developed based on those previously reported by Mukhopadhyay and Katzenstein and Ring et al. For each antibody, cases were considered positive if 10% or more tumor cells were stained. Cases with <10% staining and no focal areas of positive staining were scored as negative. Cases that were mostly negative, but contained small areas of tumor cells in which almost all tumor cells were positive were classified as focally positive. If cases were stained with either TTF-1, p63, or TRIM29 and all other antibodies in the 6-antibody panel were negative, >50% or strong diffuse staining was required for classification. Two investigators independently graded 33 cases of LADC and 42 cases of lung SqCC according to these criteria. There was a 100% concordance in their interpretations, thus indicating the reproducibility of the scoring method.

**Preparation of Mouse Monoclonal Antibody to DSG3 [BC11]**

DSG3 mouse monoclonal antibody was raised against a recombinant protein expressed in *Escherichia coli*. Splenocytes from BALB/c mice immunized with recombinant DSG3 were fused with P3x/63Ag8.653 myeloma cells to produce hybridomas. Tissue culture supernatant was screened for anti-DSG3 activity by enzyme-linked immunosorbent assay, using recombinant DSG3 as antigen. Hybridomas-producing high-affinity antibodies were subcloned by limiting dilution. An IgG1 clone, designated as BC11, was selected and further characterized by immunohistochemistry.

**Preparation of Rabbit Polyclonal Antibody to Napsin A**

Peptides corresponding to the N-terminus of Napsin A were synthesized and conjugated to keyhole limpet haemocyanin (CalBiochem, La Jolla, CA). New Zealand white rabbits were immunized subcutaneously with 0.2-mg immunoconjugates in PBS. After the initial immunization, animals were boosted 5 more times every 21 days in the same manner. The titers of the sera were evaluated by enzyme-linked immunosorbent assay on Napsin A peptide-coated plates. When the titer reached 1:10,000, the sera were purified by affinity column against immunized peptides, conjugated with goat IgG. The purified antibody was evaluated further by immunohistochemistry.

**RESULTS**

Napsin A, a novel aspartic protease, is a marker that has been frequently described in the literature over the last 10 years, but only recently has it come into its own as an important marker in the clinical setting. Studies have shown that Napsin A is a very specific marker for LADC and is superior to TTF-1 and the surfactant apoprotein. With the intent of developing a rabbit Napsin A polyclonal antibody optimized for lung cancer diagnosis, a rabbit polyclonal antibody was generated in house based on positive staining for LADCs and negative staining for lung SqCC. A side-by-side comparison of the in house rabbit Napsin A polyclonal antibody with a mouse Napsin A monoclonal antibody [TMU-Ad 02], and with a commercially available rabbit Napsin A polyclonal antibody was done on various lung cancers and normal and neoplastic tissues (data not shown). The in house rabbit Napsin A polyclonal antibody and the mouse Napsin A mouse monoclonal antibody were 88% sensitive and 100% specific and 84% sensitive and 100% specific for LADC, respectively (data not shown). When compared with the commercially available rabbit Napsin A polyclonal antibody, the in house rabbit Napsin A polyclonal antibody provided equal sensitivity, sharper staining, and was more specific in that it was negative for colon cancers (data not shown). Most importantly, the
inhomogeneous rabbit Napsin A polyclonal antibody facilitates its combination with mouse DSG3 in an antibody cocktail for multiplex staining.

The inhouse generated monoclonal antibody DSG3 [BC11] was evaluated on TMAs of LADC and lung SqCC. A high percentage of lung SqCC were positive and all LADCs were negative (data not shown).

Subsequent to the pilot study of 15 antibodies previously reported to have utility in lung cancer classification, 6 antibodies were selected for further evaluation based on their sensitivities and specificities and their potential for definitive classification of the greatest number of LADC and lung SqCC cases, when used in combination: Napsin A, DSG3, TTF-1, CK5, p63, and TRIM29.

The 6-antibody panel was evaluated for sensitivity and specificity on 95 cases of SqCC and 115 cases of LADC. The results for each individual antibody are summarized in Table 1. However, the greatest utility of this antibody panel is observed when combinations of antibodies are used. For example, Napsin A provided 87% sensitivity and 100% specificity for LADC, whereas TTF-1 provided 69% sensitivity and 94.7% specificity for LADC. Together, these 2 antibodies (positive staining with Napsin A and/or TTF-1) provided increased sensitivity for LADC (91.3%) and 100% specificity, when both Napsin A and TTF-1 were positive, or either were positive and 2 or more squamous cell markers were negative (Table 2).

Similarly, the combination of DSG3 and CK5 is particularly valuable for the identification of lung SqCC. Although DSG3 and CK5 are both 100% specific for SqCC, they have sensitivities of 85.3% and 86.4%, respectively, when used individually. When evaluated in combination (positive staining for either antibody), sensitivity for lung SqCC is increased to 92.6% and 100% specificity is maintained (Table 2). Finally, when used together, p63 and TRIM29 provided 94.7% sensitivity and 89.5% specificity for SqCC, which increased to 100% specificity in cases in which both TTF-1 and Napsin A were negative (Table 2).

The above-described combinations of antibodies specific for either LADC or lung SqCC clearly offer enhanced sensitivity and specificity over using the antibodies individually. A potentially more valuable application of the 6-antibody panel was identified using pairs of antibodies, wherein one of the antibodies is specific for LADC and the other for lung SqCC. Using this approach, an algorithm was developed based on the successive evaluation of antibody pairs that ultimately led to the classification of >93% of cases, with 100% specificity (Fig. 1).

An initial application of a Napsin A + DSG3 antibody cocktail allowed the classification of >85% of cases, with 100% specificity (Figs. 2, 3). Less than 15% of cases required further evaluation. A subsequent application of a CK5 + TTF-1 antibody cocktail increased the number of cases classified to 92%, while maintaining strong specificity (Figs. 4, 5). Finally, a few additional cases of lung SqCC can be classified by a p63 + TRIM29 antibody cocktail (Fig. 6), raising the total number of cases classified by the 6-antibody panel to >93%.

In all cases of NSCLCs, 14 of 210 (6.7%) remained unclassified by the 6-antibody panel (Table 4). Specifically, nine of 115 (7.8%) LADC and 5 of 95 (5.3%) SqCC cases were unclassified. Of note, 100% (29 of 29) of grade I cases and 96% (133 of 138) of grade II cases were able to be classified using this method. Although slightly lower, 87% (60 of 69) of grade III tumors were also successfully classified using the 6-antibody panel (Table 3).

**DISCUSSION**

In selecting antibodies from the pilot study for a detailed evaluation, particular attention was paid to identifying those with the greatest specificity. Of particular note in the initial screening, HMW cytokeratin [34betaE12] and CK7 both stained a high percentage of lung SqCC and LADCs, respectively; however, both provided poor specificity. MUC1 and CEACAM5 offered various sensitivities for both LADC and lung SqCC. However, both lack specificity for lung carcinomas, as they have been found to also stain a high percentage of nonpulmonary tissues.21,22

Of the antibodies selected for a detailed evaluation, TTF-1, Napsin A, and p63, or combinations thereof, have been well described in the literature and are in routine use by many histopathology laboratories.2,3,6,16,17 CK5 was selected for the panel over CK5/6 based on its superior specificity. Two new antibodies with relatively limited reports in the literature, DSG3 and TRIM29, were also included in the 6-antibody panel.

To date, 3 desmoglein subfamily members have been identified and all are members of the cadherin cell adhesion molecule superfamily.14 DSG3 is a calcium-binding transmembrane glycoprotein component of desmosomes in vertebrate epithelial cells. Microarray data have shown that DSG3 had a sensitivity and specificity of 88% and 98%, respectively, in detecting SqCC versus adenocarcinoma.15 However, only grades I to II lung cancers were evaluated in this study. Positive IHC staining with DSG3 has also been associated with longer survival for all lung cancer patients regardless of their permanent.

**TABLE 1. Six-Antibody Panel**

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>TTF-1</th>
<th>Napsin A</th>
<th>p63</th>
<th>TRIM 29</th>
<th>DSG-3</th>
<th>CK5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung ADC</td>
<td>80/115 (70.0%)</td>
<td>99/115 (86.0%)</td>
<td>13/115 (11.3%)</td>
<td>8/115 (7.0%)</td>
<td>0/115 (0%)</td>
<td>0/115 (0%)</td>
</tr>
<tr>
<td>Lung SqCC</td>
<td>5/95 (5.3%)</td>
<td>0/95 (0%)</td>
<td>84/95 (88.4%)</td>
<td>89/95 (93.7%)</td>
<td>82/95 (85.3%)</td>
<td>83/95 (87.4%)</td>
</tr>
</tbody>
</table>

ADC indicates adenocarcinoma; SqCC, squamous cell carcinoma.

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Therefore, the expression of DSG3 is not only a specific marker for lung SqCC, but has potential prognostic value.

TRIM29, a member of the TRIM protein family, is a putative transcriptional regulatory factor involved in carcinogenesis and/or differentiation. High expression of TRIM29 has been reported in gastric cancer and pancreatic cancer, correlating with enhanced tumor growth and lymph node metastasis. TRIM29 was previously used in a panel with four other antibodies for the classification of LADC versus lung SqCC; however, no data were provided for the sensitivity or specificity of the individual antibodies in the study.5

A rabbit monoclonal CK5 antibody was selected for the antibody panel over the mouse monoclonal CK5/6 antibody, based on the greater specificity of CK5 for lung SqCC. CK5 and CK5/6 showed 79% and 100% and 75% and 100% sensitivity and specificity, respectively.6,14 In this study, the rabbit monoclonal CK5 achieved 87.4% sensitivity and 100% specificity for lung SqCC. Other studies have shown that CK5/6 lacked absolute specificity

### TABLE 2. Antibody Combinations Specific for Lung Adenocarcinoma or Squamous Cell Carcinoma

<table>
<thead>
<tr>
<th>Expression of either or both antigens</th>
<th>Napsin A + TTF-1 (LADC)</th>
<th>DSG3 + CK5 (SqCC)</th>
<th>TRIM29 + p63 (SqCC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coexpression of both antigens</td>
<td>80/115 (73%)</td>
<td>80/95 (84.3%)</td>
<td>84/95 (88.4%)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>91.3%</td>
<td>92.6%</td>
<td>94.7%</td>
</tr>
<tr>
<td>Specificity</td>
<td>94.7%*</td>
<td>100%</td>
<td>88.7%‡</td>
</tr>
<tr>
<td>Unclassified</td>
<td>8.7%</td>
<td>7.4%</td>
<td>5.3%</td>
</tr>
</tbody>
</table>

*100% specific when Napsin A and TTF-1 are both positive or DSG3 and CK5 are negative.

†100% specific when Napsin A and TTF-1 are both negative.

LADC indicates lung adenocarcinoma; SqCC, squamous cell carcinoma.

### FIGURE 1. An algorithm for the classification of lung adenocarcinoma and lung squamous cell carcinoma using sequential application of 3-antibody cocktails (DSG3+Napsin A, CK5+TTF-1, p63+TRIM29).
for SqCC and stained LADCs. Furthermore, examination of mRNA expression of CK5, CK6, p63, and DSG3 in adenocarcinoma and SqCC of the lung found that CK5 and CK6 produced the highest sensitivity for SqCC, but also detected a moderate to high expression of an isoform of CK6 (CK6B) in 16 of 57 adenocarcinomas (28%), showing that CK5 expression is more specific for SqCC.14 In this same study, the expression of p63 was high in most SqCC cases, but p63 was also identified in 10 of 57 (18%) adenocarcinomas. In contrast, DSG3 was found to be more specific, with high expression in 88% of SqCC cases, compared with only one in 57 (<2%) of adenocarcinomas with detectable levels of DSG3.

The most valuable application of the 6-antibody panel is clearly the successive application of antibody cocktails in a stepwise manner for the classification of...
LADC or lung SqCC (Fig. 1). The proposed algorithm offers a simple and straightforward method for interpretation at each step. In developing such an algorithm, specificity is preferred over sensitivity in the first step, so as to ensure an unambiguous classification and avoid the need for further testing, even at the expense of leaving some cases initially unclassified. For this reason, the use of Napsin A and DSG3 as the first antibody pair is a key feature of the algorithm. The 100% specificities of these two antibodies allow for definitive classification of >85% of cases with this single antibody cocktail. Proceeding through the subsequent steps of the algorithm, antibodies of greater sensitivity are used, so as to increase the number of cases classified. CK5 and TTF-1 were selected for the second antibody cocktail due to the increased sensitivity of both. Using CK5 + TTF-1 increased the number of SqCC and LADC cases classified by 7% and 5%, respectively. Thus, with just 2 antibody cocktails, 92% of cases were classified. Most importantly, although as each successive tier adds diagnostic information to that already determined, 100% specificity can be maintained. For example, although TTF-1 is 94.7% specific alone (Table 1), it is 100% specific when DSG3 and CK5 are both negative (Table 2), precisely the scenario that would occur when applying the antibody cocktails in the sequence described here. Finally, the most sensitive antibody in the panel, TRIM29 (94.7%), combined with p63, was found to identify additional cases of SqCC (1%) not previously marked with DSG3 or CK5, supporting their inclusion as a final antibody cocktail to maximize the potential for classification. Again, due to the fact that TRIM29 + p63 is 100% specific when Napsin A and TTF-1 are both negative (Table 2), 100% specificity can be maintained, as a Napsin A negative/TTF-1 negative scenario is implied anytime one arrives at the TRIM29 + p63 cocktail in the algorithm.

As tumor grade increases, diagnosis may become more difficult; in these cases, a more extensive panel, such as that described in this study should be considered. The 6-antibody panel proved highly effective in classifying grade I and II lung cancers (100% and 95.5% of cases classified, respectively). Although grade III lung cancers seem to be more difficult to classify, 85.7% were successfully classified using this panel. When evaluating the utility of an antibody panel for LADC versus SqCC classification, the ability to classify less differentiated, higher grade tumors is a critical feature.

In a study similar to that reported here, investigators used a 5-antibody panel that included CK5/6, TRIM29, LAT-1, CEACAM5, and MUC1 for the classification of LADC and SqCC. Using a weighted mathematical formula based on scoring results, this panel of antibodies achieved 85% and 88% classification for LADC and lung SqCC, respectively, while leaving 12.8% of the total number of cases unclassified (Table 4). In contrast, using a routine interpretation familiar to all pathologists, the 6-antibody panel reported here provided 91.3% and 94.7% classification for LADC and lung SqCC, respectively, resulting in fewer unclassified cases (7.1%).

The high percentage of classification achieved with this 6-antibody panel compared with those previously reported may be attributable to several major factors, including the high specificity of DSG3 and sensitivity of TRIM29; the development of a rabbit polyclonal Napsin A specifically for lung diagnosis; and a highly sensitive detection method that included the use of a pressure device and a modified citrate buffer formulation (DIVA). Furthermore, the titer of each antibody was optimized in custom diluents, thus providing increased specificity (Supplementary Table 2). Careful selection of antibodies with complementary sensitivities and specificities best suited for definitive classification of the greatest number of cases, in combination with retrieval and detection protocols that optimize staining sensitivities, provides a superior method for the classification of LADC and lung SqCC.

The 6 antibodies selected from the pilot study based on their sensitivities and specificities form a useful panel for the classification of LADC and SqCC. Antibodies to Napsin A and DSG3 specifically produced and optimized for lung cancer diagnosis form the foundation of the panel. Using the 6 antibodies in cocktails of 6 antibodies (one specific for LADC and the other for lung SqCC) in a straightforward algorithm based on routine interpretation.

<table>
<thead>
<tr>
<th>Grades</th>
<th>No. Cases</th>
<th>Percent Classified</th>
<th>Percent Unclassified</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>206 (%)</td>
<td>101/206 (97.9%)</td>
<td>5/206 (2.1%)</td>
</tr>
<tr>
<td>II</td>
<td>109 (52%)</td>
<td>104/109 (95.5%)</td>
<td>5/109 (4.5%)</td>
</tr>
<tr>
<td>III</td>
<td>60 (33%)</td>
<td>58/60 (96.7%)</td>
<td>2/60 (3.3%)</td>
</tr>
</tbody>
</table>

**TABLE 3. Tumor Grade Correlation**

**FIGURE 6.** Lung squamous cell carcinoma stained with p63 (DAB)+TRIM29 (Fast Red).
provides a convenient method for definitive classification of LADC and SqCC. Using antibody cocktails with the proposed algorithm offers the additional advantage that when challenged by limited tissues, a high majority of lung cancer cases can be classified using only 1 or 2 slides. To the best of our knowledge, this staining panel provided the highest sensitivity and specificity than that has been previously reported.

**REFERENCES**


**TABLE 4. Classification Using the 6-Antibody Panel Compared With the Antibody Panel of Reference**

<table>
<thead>
<tr>
<th>Lung Carcinoma</th>
<th>Percent Classified</th>
<th>Reference 5</th>
<th>Percent Unclassified</th>
<th>Reference 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenocarcinoma</td>
<td>91.3%</td>
<td>85.4%</td>
<td>8.7%</td>
<td>14.6%</td>
</tr>
<tr>
<td>SqCC</td>
<td>94.7%</td>
<td>88.8%</td>
<td>5.3%</td>
<td>11.2%</td>
</tr>
<tr>
<td>Cases ± adenocarcinoma/SqCC</td>
<td>92.9%</td>
<td>87.2%</td>
<td>7.1%</td>
<td>12.8%</td>
</tr>
</tbody>
</table>

SqCC indicates squamous cell carcinoma.