Immunohistochemical Subtyping of Nonsmall Cell Lung Cancer Not Otherwise Specified in Fine-Needle Aspiration Cytology

A Retrospective Study of 103 Cases With Surgical Correlation

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BACKGROUND: Histopathological subtyping of nonsmall cell lung cancer (NSCLC) is currently relevant in treatment decision because of a differential activity of specific therapeutic agents. Immunohistochemistry highlights cell differentiation lineages and, in this study, it was applied to maximize the proportion of accurately subtyped NSCLC not otherwise specified (NOS) on fine-needle aspiration cytology (FNAC) samples. METHODS: Cell blocks from 103 FNAC samples with a morphological diagnosis of NSCLC-NOS were immunostained for cytokeratin (CK) 7, CK5, TTF1, and p63, whereas p40, napsin A (Naps-A), and desmocollin-3 (DSC-3) were only assessed in a subgroup of cases with discordant (CK7 and TTF1+ for nonsquamous, CK5 and p63+ for squamous) findings. Results were correlated with surgical specimens evaluated by morphology alone. RESULTS: Thirty-seven (36%) tumors with CK7/TTF1+ and CK5/p63+/CK5/p63− corresponded to 35 cases of adenocarcinoma (ADC) and 2 cases of large cell carcinoma, whereas 9 (9%) cases with the reverse immunoprofile were squamous cell carcinoma (SQCC) at surgery (P < .001). Although the remaining 57 cases had different marker combinations, a correlation was found with ADC histology for TTF1+ samples (independent of other markers) and with SQCC for p63+/TTF1− immunophenotype (P < .001). p40 was never expressed in p63+ ADC, whereas Naps-A was restricted to ADC and SQCC lineage. The percentage of unclassified NSCLC-NOS decreased from 36% to 14%. Combinations of 2 antibodies (TTF1/DSC-3 or p63/Naps-A) in the same section allowed diagnostic optimization in scant cytological samples. CONCLUSIONS: This 4-antibody panel approach may contribute to refine lung cancer classification in FNAC cell blocks, remarkably reducing the NSCLC-NOS diagnostic category. Cancer 2011;117:3416–23. © 2011 American Cancer Society.

KEYWORDS: nonsmall cell cancer, diagnosis, fine-needle aspiration cytology, tumor histotype, markers, immunohistochemistry.

In lung cancer, the simple dichotomy between small cell lung cancer (SCLC) and nonsmall cell lung cancer (NSCLC) is no longer acceptable based on the recent clinical evidence of specifically tailored systematic treatments according to different lung cancer histotypes. Consequently, a mandatory need for improving tumor subtyping is emerging. Unfortunately, this effort may be challenged by small sample size of primary or metastatic lung tumors obtained through fine-needle aspiration cytology (FNAC) or tiny bronchoscopic biopsies, in which the precise tumor definition may be hampered because of scant viable cells and/or poor tumor differentiation. The current World Health Organization classification of lung tumors states that histological typing is largely based on hematoxylin and eosin (H & E) staining, and specific histotypes (eg, large cell carcinoma [LCC]) can be adequately assessed only when surgical samples are available. Therefore, there is an increasing need for additional diagnostic techniques such as immunohistochemistry (IHC).

Several recent studies have demonstrated that different panels of IHC markers may be useful to define a specific cell lineage—namely, distinguishing squamous cell carcinoma (SQCC) from adenocarcinoma (ADC)—not only on surgical
material,7-9 but also on cytology10 or biopsy samples.11-14 However, a fraction of poorly differentiated or undifferentiated NSCLC still remains.3

We retrospectively considered a consecutive series of patients with early NSCLC who were candidates for surgery at the time of the diagnosis for whom preoperative FNAC were only available. We investigated the reliability of a panel of IHC markers to optimize the pathological diagnosis in cytological material. A diagnostic concordance rate between cytological samples and the corresponding surgical specimens was demonstrated in half of cases with a concordant immunophenotype. In the other half of cases, in which a less clear immunophenotype was obtained, an algorithm considering sequential IHC markers was proposed, resulting in an improved diagnostic accuracy.

**MATERIALS AND METHODS**

**Case Selection**

From the databases of the Pathology Divisions at San Luigi Hospital in Orbassano-Turin and Forlanini Hospital in Rome, Italy, 1072 cases recorded over a 5-year period (January 2004 through December 2008) and coded as transthoracic FNAC of primary lung cancer were collected retrospectively. Among these, 61 cases were reported as SCLC by morphology and IHC, and 642 cases were classified as SQCC or ADC by morphological findings alone. The remaining 369 cases (36% after excluding SCLCs) were diagnosed as NSCLC-NOS in the absence of clear signs of either squamous or glandular differentiation, and 112 of these 369 patients subsequently underwent surgical resection at the same institutions and had available cell block material (a figure that is in line with the expected percentage of 20%-30% operable cases). Upon revision of the original H & E- or Giemsa-stained smears and cell block slides, 9 cases were excluded after they were reclassified as poorly differentiated SQCC or ADC. The 103 remaining cases were labeled with anonymous codes for clinical correlations and cut into serial sections for IHC staining. Surgical specimens were morphologically classified according to the 2004 World Health Organization classification5 criteria (and not on the basis of immunoprofile) and included 64 ADCs, 24 SQCCs, 1 adenosquamous carcinoma, 6 LCCs, and 8 sarcomatoid carcinomas. The study was approved by the Institutional Review Board of the San Luigi Hospital, Turin, Italy.

**Conventional Cytology Stains and IHC**

FNAC sampling had been performed using 21- to 23-gauge fine needles: 1 smear was stained with H & E for adequacy evaluation and another was air-dried for Giemsa stain. Any extra material was placed into 95% ethanol-containing vials, centrifuged, and processed for cell block and paraffin embedding. Five-micron-thick serial sections were collected onto charged slides, dewaxed, rehydrated in pH 7.5 buffer, and processed for standard IHC staining. Briefly, after blocking endogenous peroxidase activity in 0.3% hydrogen peroxyde and methanol solution for 15 minutes, 5-μm-thick cell block sections were reacted for 40 minutes at room temperature with cytokeratin (CK) 7 (dilution 1/200; Neomarkers-Thermo Scientific, Fremont, CA), CK5 (dilution 1/50; Dako Cytomation, Glostrup, Denmark), TTF1 (dilution 1/100; Dako Cytomation), p63 (dilution 1/200; Neomarkers-Thermo Scientific), p40 (dilution 1/800, Calbiochem, Nottingham, UK), napsin A (Naps-A) (dilution 1/4000 for 40 minutes, ARP, Belmont, MA), and overnight at 4°C with desmocollin-3 (DSC-3) (dilution 1/30, PROGEN Biotechnik, Heidelberg, Germany). Slides were then incubated in a detection kit (EnVision Plus HRP, Dako Cytomation) according to the manufacturer’s instructions, developing peroxidase activity with 3-3’-diaminobenzidine. Antigen retrieval was performed in a pressure cooker for 5 minutes at 125°C followed by a quick 10-second step at 90°C using pH 8.0 ethylenediamine tetra-acetic acid buffer for all primary antibodies, and pH 6.6 citrate buffer for DSC-3. Finally, slides were counterstained with hematoxylin, dehydrated, and mounted. The specificity of all immunoreactions was double-checked by substituting the primary antibody with a nonrelated isotypic mouse immunoglobulin at a comparable dilution, and with normal serum alone. Immunoreactivity of normal bronchioloalveolar epithelium was used as an internal positive control for glandular markers and p63.

**Combined Immunohistochemistry**

Combinations of 2 antibodies with complementary and non-overlapping cell staining (TTF1/Naps-A or DSC-3 and p63/DSC-3 or Naps-A) were also tested on selected cases with the purpose of maximizing results of cell lineage assessment in poorly cellular samples. The 2 individual primary antibodies were simultaneously applied to the same cell block section in a single solution using the same final dilution and protocol procedure detailed above. All other immunostaining steps (dewaxing, rehydration, peroxidase blockage, antigen retrieval, detection,
development, and counterstaining) were performed as detailed above for conventional IHC stain.

**Analysis of Immunohistochemical Findings**

IHC was scored as either negative, if no staining was found, or positive, considering any weak or strong stain with no cutoff level of expression, based on the expected nuclear or cytoplasmic reactivity of each marker in morphologically confirmed neoplastic cells. With regard to the panel of ADC markers (TTF1 and CK7) or SQCC markers (p63 and CK5) tested in all 103 cases, the immunohistochemical findings were interpreted as follows: 1) a combined CK7 and TTF1 reactivity in the absence of p63 and CK5 expression was considered supportive of ADC diagnosis; 2) the opposite marker combination favored a SQCC diagnosis; 3) tumors having any other marker combinations (including quadruple negative cases) were further assessed by additional markers for SQCC (DSC-315-16 and p4017) or ADC (Naps-A8) differentiation. A diagnostic algorithm was proposed in order to maximize the diagnostic accuracy of this panel of IHC markers on cell block samples, compared to the histological diagnosis on surgical specimens.

**Statistical Analysis**

A chi-square test was performed to assess the significance of the correlation between the FNAC diagnoses and the corresponding postsurgical histological diagnoses. The Cramer V index was used to measure the strength of the cytology-histology concordance. The specificity (SP), sensitivity (SE), negative and positive predictive values, and diagnostic accuracy rates of all markers under evaluation were calculated and reported in Table 1, along with the corresponding 95% confidence intervals. The level of significance was set at $P = .05$.

### RESULTS

#### Conventional Cytology IHC

The 103 FNAC samples originally diagnosed as NSCLC-NOS on a morphological ground were categorized into several groups based on the immunoprofile obtained with the markers TTF1, p63, CK5, and CK7 (Figure 1). One group included 46 FNAC cases (44.7%) that were consistent with a diagnosis of SQCC (CK7+/TTF1− and CK5/p63+, 9 cases) or of ADC/LCC (CK7+/TTF1+ and CK5/p63−, 37 cases), as confirmed by the corresponding surgical specimen diagnoses: these included all 9 SQCCs, 35 ADCs, and 2 LCCs displaying an immunoprofile consistent with glandular differentiation (TTF1/CK7+) (Cramer V index = 1; $P < .001$ [chi-square test]).

The other groups of tumors included all the remaining 57 cases (55.3%) displaying variable marker combinations on FNAC samples (1 case having a quadruple-negative phenotype). On surgical pathology specimens, the final H & E diagnosis in this group was 29 ADCs, 15 SQCCs, 1 adenosquamous carcinoma, 4 LCCs, and 8 sarcomatoid carcinomas (Cramer V index = 0.49; $P < .001$ [chi-square test]). In these 57 cases, the observed possible immunophenotypic combinations were analyzed in different steps (Figure 1).

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**Table 1.** Distribution of Immunohistochemical Markers in 103 Fine-Needle Aspiration Cytology Samples According to Surgical Histological Diagnosis and in Selected Cases Originally Showing a Heterogeneous Immunophenotype

<table>
<thead>
<tr>
<th>Histological Diagnosis</th>
<th>All cases (N=103)</th>
<th>Selected cases (n=57)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADC (n=66)</td>
<td>SQCC (n=24)</td>
<td>LCC-SARC (n=12)</td>
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<tr>
<td>ADC (n=66)</td>
<td>SQCC (n=24)</td>
<td>LCC-SARC (n=12)</td>
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<td>ADC (n=66)</td>
<td>SQCC (n=24)</td>
<td>LCC-SARC (n=12)</td>
</tr>
<tr>
<td></td>
<td>SP (95% CI)</td>
<td>SE (95% CI)</td>
</tr>
<tr>
<td>TTF1+ (n=54)</td>
<td>51</td>
<td>0</td>
</tr>
<tr>
<td>p63+ (n=45)</td>
<td>13</td>
<td>24</td>
</tr>
<tr>
<td>CK7+ (n=91)</td>
<td>66</td>
<td>15</td>
</tr>
<tr>
<td>CK5+ (n=25)</td>
<td>3</td>
<td>19</td>
</tr>
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<tr>
<th>Selected cases (n=57)</th>
<th>ADC (n=29)</th>
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<td>ADC (n=29)</td>
<td>SQCC (n=15)</td>
<td>LCC-SARC (n=12)</td>
<td></td>
</tr>
<tr>
<td>Naps-A+ (n=12)</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DSC-3+ (n=15)</td>
<td>0</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>p40+ (n=20)</td>
<td>1</td>
<td>15</td>
<td>1</td>
</tr>
</tbody>
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Abbreviations: ADC indicates adenocarcinoma; SQCC, squamous cell carcinoma; ADSQ, adenosquamous carcinoma; LCC, large cell carcinoma; SARC, sarcomatoid carcinoma; SP, specificity; CI, confidence interval; SE, sensitivity; PPV, positive predictive value; NPV, negative predictive value; DA, diagnostic accuracy; Naps-A, napsin-A; DSC-3, desmocollin-3.
TTF1 reactivity was considered at first. This was found in 17 out of 57 (29.8%) tumors (independent of CK5, CK7, or p63 reactivities) that were eventually diagnosed as ADC (14 cases, 82.4%), LCC (2 tumors), and sarcomatoid carcinoma (1 tumor of the pleomorphic type with an ADC component). An unexpected presence of CK5 and p63 was detected in 3 and 12 cases, respectively. With regard to the latter marker, none of the 12 p63+ cases reacted with p40, which is specific for the truncated p63 isoforms present in squamous epithelia. These findings confirmed the role of TTF1 as a strong predictor of glandular phenotype with a high sensitivity (SE = 0.77) and maximum specificity (SP = 1), when squamous carcinoma were compared with nonsquamous cancer (ADC and LCC).

Subsequently, after excluding TTF1+ cases, 40 tumors remained, 22 (38.6%) of which were p63+ (any CK5 or CK7), corresponding to a final diagnosis of SQCC and adenosquamous carcinoma in 15 tumors and 1 tumor, respectively (overall 72.7%); the remaining cases were 5 sarcomatoid carcinomas and 1 ADC (p63/CK7+) having an apparently aberrant p63 expression (being p40 stain negative, see above). CK5 was coexpressed in 60% of cases (13/22)—all of which were histologically proven SQCC—and, unexpectedly, CK7 immunoreactivity was observed in all but 1 SQCC case (thus limiting the CK7 diagnostic role as an ADC marker in p63-expressing cases).

Finally, excluding the 17 TTF1+ and the 22 p63/CK5+ cases, 17 (29.8%) other tumors expressed CK7 only. These tumors eventually turned out to be ADC (14 cases, 82.4%), LCC (2 cases), and sarcomatoid carcinoma (1 tumor). The single remaining quadruple-negative tumor was a sarcomatoid carcinoma devoid of any differentiation markers, a finding confirmed even in the surgical specimen (Table 1).

The same 57 cases evaluated in this stepwise diagnostic algorithm were also assessed for additional markers. Naps-A was expressed in 11 out of 29 ADCs, 4 of which were TTF1+ and had a CK7-only phenotype, supporting its role as an additional glandular lineage diagnostic marker. Conversely, the histologically proven squamous (or adenosquamous) carcinomas expressing squamous cell markers (p63 or CK5), also displayed membranous DSC-3 reactivity in 13 out of 16 cases. This result apparently did not improve the other squamous marker performance. However, DSC-3 reactivity was never detected in histologically proven ADCs at surgery, including those that variably expressed p63 or CK5. Finally, the role of p40 has already been mentioned: although positive in all SQCCs, p40 was negative in all but 1 ADC, which displayed a focal and faint immunostaining (Table 1).

**Combined Cytology IHC**

In selected cases, IHC with antibody combinations in the same section allowed identification of glandular versus squamous lineages in poorly cellular cases in which serial sectioning was difficult to obtain. In general, IHC using a combination of 2 different-by-lineage, specific antibodies

![Figure 1. Diagnostic algorithm based on the different immunoprofile combinations in 103 fine-needle aspiration cytology cases originally diagnosed as nonsmall cell lung cancer “not otherwise specified” is shown. On the left side, 46 tumors had a concordant profile (with probable cytological diagnoses) compared with the corresponding surgical histological diagnoses. On the right side, the remaining 57 tumor cases with any other profile, were divided according to the different marker combinations (considering at first the tumors expressing TTF1, then those having p63 reactivity, and then the remaining CK7+ only cases and a single case of completely unreactive sarcomatoid carcinoma), the favorable cytological diagnoses and the corresponding surgical diagnoses. Gray arrows: immunoprofile combinations. Green boxes: cytological diagnoses. White boxes: corresponding surgical histological diagnoses. Abbreviations: ADC indicates adenocarcinoma; ADSQ, adenosquamous carcinoma; LCC, large cell carcinoma; NSCLC-NOS, nonsmall cell lung cancer not otherwise specified; sarcom Ca: sarcomatoid carcinoma; SQCC, squamous cell carcinoma.](image)
was easier to interpret than homogeneous-by-lineage antibodies (TTF1 and Naps-A or p63 and DSC-3), although the results were largely dependent on the cellularity and cell arrangements of each single case.

**DISCUSSION**

This study demonstrated that a limited panel of IHC markers may reliably refine NSCLC histotyping or, at least, provide the most likely differentiation lineage of poorly differentiated lung cancers originally diagnosed as “NSCLC-NOS” in FNAC specimens.

The proposed antibody panel includes IHC markers that are well-known, commercially available, inexpensive, commonly used, and standardized in any pathology laboratory and may be easily applied to either bronchial biopsies or cytological bronchial specimens. Other investigators have used similar markers, especially TTF1 and p63, and detected a correlation with histological specimens. A panel of immunophenotypic markers as well as PAS stain were able to address the correct tumor histotype in the majority of the investigated cases, although the reference standard for the final histological diagnosis was not based on surgical resected tissue. The present FNAC study was limited to surgically resected cases to provide an accurate comparison with the final tumor subtyping. In addition, we only analyzed undifferentiated NSCLC on FNAC samples, because cytological material containing well-differentiated squamous or glandular tumors could be more easily classified. We observed that a similar panel of markers that included TTF1, p63, CK5, and CK7, was helpful to reduce the percentage of unclassified FNACs, suggesting the most probable histotype of tumors. Considering the whole series of NSCLC at our institutions, it appears that 36% of cases were morphologically classified as NSCLC-NOS, the other 64% being correctly classified by morphology alone as SQCCs or ADCs (data not shown). Using a 4-marker panel, we observed that slightly less than half of such unclassified tumors (16% of the total) had an immunophenotype that was fully predictive of nonsquamous (ADC/LCC) (CK7/TTF1+ and CK5/p63−) or SQCC (CK7/TTF1− and CK5&p63+) differentiation lineages, according to the postsurgery histological diagnosis. In the other half of NSCLC-NOS cases, the immunoprofile was more heterogeneous, even though the histotype could still be predicted with an acceptable accuracy (72.7%-82.4%) with special regard to TTF1+ cases in a fraction of NSCLC cases.

In the present study, TTF1 was the most specific marker of ADC, although its sensitivity did not exceed 77%, which well compares with previously reported
values. Such sensitivity values are usually reported from surgical series; therefore, these figures may further decrease in FNAC samples or bronchial biopsies, due to focal TTF1 expression in some cases of ADC. This marker has been largely used in biopsy or cytological series, because the nuclear reactivity is readily apparent even in poorly cellular samples and its specificity is high. In the current study, after the exclusion of the fully concordant phenotype cases, TTF1 was positive in approximately one-third of the remaining cases (irrespective of the other marker reactivities), and all of them were ADC or LCC with glandular marker expression (the category also termed “nonsquamous histology” in clinical trials).

Such an IHC approach allowed us to correctly subclassify up to 22% of cases, thus reducing the percentage of undetermined diagnoses to 14% (Figure 3). Such undetermined tumor types had no TTF1 reactivity and variably expressed the other markers. Although these were less sensitive and specific, they were nevertheless relatively accurate in identifying an histotype when compared with the final surgical diagnosis.

In agreement with previously published reports, the majority of SQCCs had a diffuse and strong p63 nuclear expression, but up to 15% of ADCs also displayed some p63 reactivity. The widely used 1A4 monoclonal antibody to p63 recognizes all p63 isoforms, whereas p40 polyclonal antibody is specific for non-transactivating isoforms. It could be argued that some p63+/ADC according to panisoform-specific antibodies may express transactivating p63 isoforms, which are not identified by p40 antibody, as indicated by one of the current authors (G. P.) in a preliminary report. This latter marker seems therefore superior to p63 in the recognition of SQCC.

In the absence of TTF1 and p63, CK5 or CK7 cannot define a histotype when used alone, so additional immunohistochemical markers were explored. Naps-A expression confirmed the glandular lineage in almost half of TTF1+ cases, with a high specificity rate (SP = 1). Naps-A was expressed in 4 TTF1− cases (that had CK7 expression only), supporting its role as an additional ADC marker. In TTF1− tumors, coexpression of DSC-3 and p63 supported a diagnosis of SQCC (confirmed in 72.7% of cases at surgery), and proved highly specific for SQCC (SP = 1), irrespective of p63/p40 reactivity. DSC-3 was never seen in association with TTF1, with the exception of the adenosquamous carcinoma having a composite differentiation.

Correlating the overall accuracy between FNAC IHC findings and the corresponding surgical specimen diagnoses in the 90 ADC/LCC or SQCC cases (and the 2 LCCs having both TTF1 and CK7 expression), the correct diagnosis was suggested in FNAC samples with a complete concordance (Cramer V index = 1; P < .001 [chi-square test]). The other 13 cases, showing an ambiguous or fully negative phenotype on FNAC, were combined or undifferentiated tumors in the corresponding surgical specimen, with a histological diagnosis of adenosquamous carcinoma (1 case), LCC (4 cases), or sarcomatoid carcinoma (8 cases).

In addition, the current study was based on cell block samples containing sufficient material to provide up to 7 additional serial sections for IHC. Because this is not the rule in daily practice and poorly cellular cell block samples do occur, a 2-antibody combination was tested on the same section. We observed that nuclear markers (p63 or TTF1) combined with the opposite lineage cytoplasmic or membrane marker (Naps-A or DSC-3, respectively) yielded the best results in identifying tumor differentiation, compared with the cytokeratins containing combinations.
In conclusion, our data show that an appropriate panel of IHC markers allow the correct classification in 86% of cases originally diagnosed as NSCLC-NOS on the basis of morphology alone. When the immunoprofile fulfilled the expected criteria for glandular and squamous cell differentiation, the final tumor classification on surgical specimens was anticipated with a high rate of sensitivity and specificity. In the case of a heterogeneous immunoprofile, it was demonstrated that TTF1 is the most robust predictor of ADC, truncated p63 is the most robust predictor of SQCC when TTF1 was negative. CK7 and CK5 are preferentially (but not exclusively) expressed in ADC or SQCC, respectively, as expected, even in FNAC samples, but other markers are needed to support their diagnostic potential. In this respect, Naps-A or DSC-3 are useful specific (though less sensitive) new candidates for favoring a diagnosis of ADC or SQCC, respectively.

These findings, combined with other recent data on biopsy and cytology samples, indicate that a correct lung cancer histotype assessment can be obtained in a large fraction of cases, even those cases with a limited amount of available neoplastic cells. In addition, the current study indicates that the percentage of indeterminate diagnoses (NSCLC-NOS) can be reduced to 10%-15%, even in case series specifically analyzing poorly differentiated carcinomas, thus defining a role for immunoprofiling in NSCLC to support histology-driven selection of systemic therapy.

CONFLICT OF INTEREST DISCLOSURES
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