Recommendations for immunocytochemistry in lung cancer typing: An update on a resource-efficient approach with large-scale comparative Bayesian analysis

Hatice Elmas1 | Roland Diel2,3 | Binnur Önal4 | Guido Sauter1 | Florian Stellmacher5 | Lutz Welker1

Abstract

Objectives: The majority of lung cancer cases are of advanced stage and diagnosis is usually made using minimally invasive small biopsies and cytological specimens. The WHO 2015 classification recommends limiting immunocytochemistry (ICC) to lung cancer typing and molecular testing drives for personalised therapies. An algorithm using Bayes' theorem could be useful for defining antibody profiles. This study aims to assess the impact of different antibody profiles for cytological samples on the accuracy of lung cancer typing with a large-scale Bayesian analysis.

Methods: A retrospective examination of 3419 consecutive smears and/or cytospins diagnosed over 2011-2016 found 1960 primary lung cancer tumours: 972 adenocarcinomas (ADC), 256 squamous carcinomas (SQC), 268 neuroendocrine tumours (NET), and 464 non-small cell cancer-not otherwise specified (NSCC-NOS). The a priori and a posteriori probabilities, before and after ICC using antibodies singly or in combination, were calculated for different lung cancer types.

Results: TTF-1 or CK7 alone improved the a posteriori probabilities of correct cytological typing for ADC to 86.5% and 95.8%, respectively. For SQC, using p40 (∆Np63) or CK5/14 together with CK5/6 led to comparable results (78.3% and 90.3%). With synaptophysin or CD56 alone, improvements in a posteriori probabilities to 87.5 and 90.3% for the correct recognition of NET could be achieved.

Conclusions: Based on morphological and clinical data, the use of two antibodies appears sufficient for reliable detection of the different lung cancer types. This applies to diagnoses that were finalised following ICC both on a clinical or cytological basis and on a histological basis.

KEYWORDS
Bayes theorem, cytology, immunocytochemistry, lung cancer, tumor typing
Lung cancer is the most common cause of death from cancer worldwide. Respiratory cytology has continued to dominate in the initial evaluation and diagnosis of lung cancer since Dr Papanicolaou’s era from the 1940s. However, more recent advances in molecular pathology diagnostics and the development of targeted therapy have significantly expanded the quantitative and qualitative spectrum of biopsy diagnostics. The potential offered by these advances has, in turn, brought the large number of previously excluded patients with advanced tumours to the forefront of scientific interest. These changes led the World Health Organization (WHO) to develop the concept of “small biopsies and cytology” in 2015. This raises two questions: first, the validation of such obtained results, and second, their ranking in the context of subtle clinical findings (imaging, etc.) compared with final histologically confirmed diagnoses on surgically obtained specimens. Accurate typing and subtyping of lung cancer is critical for several reasons: Therapeutically significant EGFR mutations and other multiple additional molecular targets, including ALK, ROS1, and RET rearrangements, BRAF and MET exon 14 splice site mutations, occur preferentially in lung adenocarcinoma (ADC). ADC histology is a strong predictor for improved outcome with pemtretrexed therapy compared to squamous cell carcinoma (SQC), and potential life-threatening haemorrhage may occur in patients with SQC who receive bevacizumab. Lastly, between neuroendocrine tumour (NET) subtypes, such as carcinoid or small cell lung carcinoma (SCLC) and large cell neuroendocrine carcinomas (LCNEC), we find relevant differences regarding treatment options and prognoses. The increased therapeutic relevance of typing lung carcinomas poses a particular challenge for cytological diagnostics.

Therefore, it is necessary to provide clinicians and (cyto-)pathologists with serious answers to the question of with what probability are different immunocytochemistry (ICC) marker combinations suitable for correctly identifying primary lung cancer types.

This study aims to assess the influence of different staining protocols on diagnostic safety of primary lung cancer typing in a large series of small biopsies and cytology specimens. Clinically/cytologically and histologically confirmed diagnoses will be compared.

2 | MATERIALS AND METHODS

2.1 | Case collection

The database of the Cytology laboratory at the LungenClinic Grosshansdorf was queried for all consecutive immunocytoologically examined samples over a period of 68 months (January 2011 to October 2016, Figure 1). The search yielded 3419 specimens from 2326 patients with 1960 exclusively primary lung tumours, consisting of 1197 fine needle aspiration (FNA) samples (endobronchial ultrasound, n = 512; transoesophageal endoscopic ultrasound, n = 47; somatex spiral needle, n = 217; yale needle, n = 420; rigid transbronchial needle, n = 1), along with touch preparations (n = 436), brush cytology (n = 116) and pleural effusion (n = 212) specimens. The resulting diagnoses included examples of all NET subtypes: SCLC (n = 166), LCNEC (n = 85), atypical carcinoids (AC, n = 1), and typical carcinoids (TC, n = 10). Tumours of extrapulmonary origin were excluded.

The endpoint of the study was the last (final) diagnosis of the 3419 specimens before the initiation of therapy. These final diagnoses were compared with the final histologically confirmed diagnoses on surgically obtained specimens. The scale of the study is shown in Figure 1.
diagnoses consisted of 858 and 1102 cases based on 1495 clinical/cytological findings and 1924 histological results, respectively (Table 1). Cytological tumour typing was based on the WHO 2015 classification for lung tumors.5

2.2 Immunocytochemistry (ICC)

Following standard Giemsa staining on direct smears and cytospin slides, suitable slides were chosen for ICC. All of the ICC stains were performed manually using unstained air-dried specimens.13 Up to three different antibody stains per slide were applied (Figure 2). The manually immunostaining patterns of cytokeratin7 (CK7, clone OVTL12/30), thyroid transcription factor1 (TTF-1, clone SPT24), p40 (ΔNp63, polyclonal), CK5/6 (clone D5/16B4), CK5/14 (clone XM26/SFI-3), neural cell adhesion molecule 1 (CD56, clone I23C3.D5), and synaptophysin (Syn, clone SPI I) were correlated to obtain an optimum result. There are considerable morphological differences between subtypes of NET, but no differences were found in their ICC expression patterns (Figure 3).6,14-16

For the qualitative assurance of immunohistochemical staining results, suitable external or internal positive and negative controls were included for all histological sections and cytological samples.10,17 As pretreatment, CK5/6 antibody specimens were preheated in citrate buffer at pH 8.0, whereas with the exception of CK5/14, all other specimens were preheated in citrate buffer at pH 6.0.13

2.3 Statistical analysis

The following data were collected retrospectively from electronic medical records of LungenClinic Grosshansdorf for the purpose of statistical analyses: patient age, gender, tumour location (lung tumour), type of procedure (touch preparation, FNA, brush cytology, pleural effusion), and final cytopathological and/or histopathological diagnosis. The final diagnosis was based on a multi-disciplinary approach, including clinical-radiological, histopathological, and clinical-cytopathological considerations, along with the immunohistochemistry (IHC) and ICC results.

A Bayesian analysis was used to calculate the a priori probabilities of the individual types and subtypes in the specimens.18,19 These values were compared with the ICC diagnosis achieved when using single or multiple antibodies. Finally, these results were supplemented by a calculation of the sensitivity, specificity, and the negative predictive value for different marker constellations.17,20

Using a large-scale Bayesian analysis of the data, the gain in the probability of individual hypotheses before (a priori) and after (a posteriori) the use of an antibody in the individual types of lung cancer was calculated or specifically estimated.19,20

Statistical analysis was performed using the Fisher exact test, and all P values smaller than 0.05 were considered significant.18,19,21

3 RESULTS

3.1 Single antibodies

Based on the routine Giemsa staining, the calculated a priori probability was 13.1% for SQC (n = 256), 49.6% for ADC (n = 972), 23.7% for non-small cell cancer-not otherwise specified (NSCC-NOS; n = 464), and 13.7% for NET (n = 268).

Table 1: Characteristics of the study

| Distribution of patients (gender, mean age, range) | n       |                  |
|---------------------------------------------------|---------|-----------------|
| Female                                            | 811     | 65.5 years      |
|                                                   |         | (range 18-92)   |
| Male                                              | 1149    | 57.1 years      |
|                                                   |         | (range 28-90)   |
| Cytotechniques                                    | 3419    |                 |
| Fine-needle aspiration (FNAs)                     | 1197    |                 |
| Touch preparations                                | 435     |                 |
| Effusions                                         | 212     |                 |
| Brush cytologies                                  | 116     |                 |
|                                                   | 1960    |                 |

Table 1: Characteristics of the study

| Clinical-cytological final diagnoses (clinical diagnoses based on medical history, clinical findings, endoscopy, and imaging—X-ray, CT, PET-CT, MRI) | 858 |
|----------------------------------------------------------------------------------------------------------------------------------|-----|
| Histological confirmed final diagnosis                                                                                  | 1102|
| Histologic type                                                                                                          | 1960|
| ADC                                                                                                                         | 972 (589/383) |
| SQC                                                                                                                         | 256 (161/95)  |
| NSCC-NOS                                                              | 464 (229/235) |
| NET                                                                                                                         | 268 (123/145) |
| SCLC                                                               | 166     |
| LCNEC                                                           | 85      |
| AC                                                                                                                         | 7      |
| TC                                                                                                                         | 10     |
|                                                                                                                             | 1960   |

Abbreviations: AC, atypical carcinoid; ADC, adenocarcinoma; clc, clinical-cytological confirmed final diagnosis; h, histological confirmed final diagnosis; LCNEC, large cell neuroendocrine carcinoma; NET, neuroendocrine tumour; NSCC-NOS, non-small cell cancer-not otherwise specified; SCLC, small cell lung carcinoma; SQC, squamous cell carcinoma; TC, typical carcinoid.
Using TTF-1 or CK7 as a single antibody increased the probability of ADC from 49.6% a priori to 77.7% and 82.1% a posteriori, respectively, on positive results. In contrast, the probability for SQC decreased from 13.1% a priori to 1.4% and 1.7% a posteriori with positivity for TTF-1 and CK7, respectively. Comparable results were obtained for NSCC-NOS (TTF-1 sensitivity 87.0%, specificity 70.2%; CK7 sensitivity 91.3%, specificity 56.6%; Table 2).

Regarding p40 (ΔNp63), and CK5/6 together with CK5/14 (a combination hereafter represented as “CK5/6–CK5/14”), the probability for SQC increased from 13.1% a priori to 78.3% and 87.3% a posteriori, respectively. The a priori probability of all other lung cancer types decreases with the use of these antibodies (eg, for p40 (ΔNp63): ICC sensitivity 52.2%, specificity 93.3%; IHC sensitivity 68.7%, specificity 91.4%; Table 2).

Common to all NET is the histological feature of neuroendocrine morphology. Accordingly, the majority of NET diagnoses are already successful with routine staining procedures. CD56 or Syn positivity alone also significantly increases the a posteriori probabilities in ICC for NET (from 13.7% a priori to 90.3% with CD56, or 87.5% with Syn; Table 2). However, it is notable that NET can be partially CD56- or Syn- negative (34 of 238 cases, and 23 of 58 cases, respectively). The vast majority of non-small cell carcinomas are CD56- or Syn-negative (Table 2).

Compared with the cytological specimens, the histologically examined sections show NSCC-NOS less frequently (a priori probabilities 23.7% and 13.8%, respectively) and ADC somewhat more often (a priori probabilities 49.6% and 59.2%, respectively; Table 2).

The a posteriori probability for SQC when using CK5/6–CK5/14 on histological slides is lower than in the immunocytochemically stained slides (49.0% and 87.3%, respectively). However, this difference is not statistically significant (P > 0.71; Table 2).

On the histological sections, IHC using CD56 or Syn leads to comparable results (a posteriori probabilities of 65.6% and 66.7%, Table 2).

### 3.2 Combined analysis using two antibodies

In contrast to the use of a single antibody (eg, either TTF-1 or CK7), the combined use of two antibodies, with both results positive or with one positive and one negative, increases the probability of a correct diagnosis of ADC significantly by around 10 percentage points. Compared to the probabilities for ICC of 77.7% for TTF-1+ and 82.1% with CK7+ alone, a posteriori probabilities increase to 96.6% for TTF-1+ and CK7+ (P < 0.005), and to 91.0% for TTF-1+ and CK5/6–CK5/14− (P < 0.05); similar increases were observed from the single antibody values (83.3% for TTF-1+, 84.8% for CK7+) to those for combinations (97.3% for TTF-1+ and CK5/6–CK5/14−) for IHC specimens (Tables 2 and 3).

In comparison to ADC, there were no significant differences between the use of individual antibodies or antibody combinations in SQC (all Ps > 0.5, Tables 2 and 3).

A characteristic feature of NSCC-NOS is the lack of a recognisable differentiation. The greater the number of missing differentiating features of other tumour types, the more accurately the diagnosis of NSCC-NOS can be achieved. Thus it comes as no surprise that the results of using a single antibody on ICC are inferior compared with a combination of antibodies. Taking single results as the a priori value, the combination of two negative antibody results (for TTF-1 and CK7, CK5/6–CK5/14, or p40 (ΔNp63), all Ps < 0.001; Tables 2 and 3).

Compared to the high a priori values of NET when using a single antibody, the use of combined antibodies does not significantly improve the a posteriori probability (on ICC, 90.3% or 87.5% for the use of CD56 or Syn alone vs 85.3% for CD56 and Syn in combination; on IHC, 65.6% or 66.7% for CD56 or Syn used singly vs 75.0% for the combination, P > 0.5, Tables 2 and 3).
3.3 Combinations of more than two antibodies

Finally, the influence of combining more than two antibodies on the a posteriori probability of different lung cancer types was examined (Table 4). The combination of two positive and one negative antibody increased the a posteriori probability for ADC from 96.6% for TTF-1+ and CK7+, to 97% for TTF-1+, CK7+, and CK5/6–CK5/14– (Tables 3 and 4). The a posteriori probability of SQC when using a combination of one positive and two negative antibodies is not significantly different from a combination of only two positive antibodies (89.2% vs 95.0% or 89.0%; all P values > 0.5; Tables 3 and 4).

A combination of two negative antibodies leads in ICC to a significant improvement of the a posteriori probabilities for the presence of an NSCC-NOS (52.5%, 48.7%, 48.4%, or 35.5% for the isolated...
|      | ICC   | a priori % | TTF-1 | CK7 | CK5/6, CK5/14 | p40 (ΔNp63) | CD56 | Syn |          |          |          |          |          |          |          |          |
|------|-------|------------|-------|-----|---------------|-------------|------|-----|----------|----------|----------|----------|----------|----------|----------|
|      |       |            | Neg.  | Pos.| Neg.          | Pos.        |      |     | Neg.     | Pos.     | Neg.     | Pos.     | Neg.     | Pos.     | Neg.     | Pos.     |
| SQC  | ICC   | 13.1       | 21.2  | 1.4 | 20.8 (58)     | 1.7 (15)    | 5.2  | 19.2 (33) | 87.3 (207) | 19.2 (33) | 78.3 (36) | 4.2 (5)  | 1.8 (4)  | 2.2 (1)  |          |
|      | IHC   | 15.8       | 43.1  | 0.3 | 14.3 (3)      | –           | 2.2  | 5.0 (3)   | 49.0 (25)  | 5.0 (3)   | 55.2 (32) | –        | –        | 21.4 (3) | –        |
| ADC  | ICC   | 49.6       | 18.1  | 0.2 | 77.7 (813)    | 25.1 (70)   | 44.8 | 43.6 (75) | 5.9 (14)   | 43.6 (75) | 15.2 (7)  | 32.5 (39)| 4.0 (9)  | 17.8 (8) | 10.0 (4) |
|      | IHC   | 59.2       | 22.2  | 0.3 | 83.3 (145)    | 61.9 (13)   | 82.2 | 76.7 (46) | 23.5 (12)  | 76.7 (46) | 17.2 (10) | 61.9 (13)| 21.9 (7) | 50.0 (7) | 23.8 (5) |
| NSCC- NOS | ICC | 23.7       | 52.5  | 0.2 | 3.2 (33)      | 48.7 (136)  | 48.4 | 35.5 (61)| 5.9 (14)   | 35.5 (61)| 6.5 (3)   | 35.0 (42)| 4.0 (9)  | 28.9 (13)| 2.5 (1)  |
|      | IHC   | 13.8       | 29.2  | 0.3 | 5.2 (9)       | 19.0 (4)    | 15.6 | 13.3 (8)  | 25.9 (15)  | 13.3 (8)  | 33.3 (7)  | 12.5 (4) | 21.4 (3) | 9.5 (2)  |          |
| NET  | ICC   | 13.7       | 8.2   | 0.5 | 17.8 (186)    | 5.4 (15)    | 0.8  | 1.6 (6)   | 0.8 (2)    | 1.6 (6)   | 0.8 (2)   | –        | 28.3 (34)| 90.3 (204)| 51.1 (23)| 87.5 (35)|          |
|      | IHC   | 11.2       | 5.6   | 0.4 | 11.5 (20)     | 4.8 (1)     | –    | 2.0 (1)   | 5 (3)      | 2.0 (1)   | 1.7 (1)   | 4.8 (1)  | 65.6 (21)| 7.1 (1)  | 66.7 (14)|          |
| Total| ICC   | 100.0      | 674   | 147 | 279           | 895         | 366  | 172       | 237        | 172       | 46        | 120      | 226      | 45       | 40       |
|      | IHC   | 100.0      | 72    | 174 | 21           | 46          | 45   | 60        | 58         | 60        | 58        | 21       | 32       | 14       | 21       |

Note: The a priori probability shows computation of existing data before performing an investigation. The a posteriori probability is for the diagnosis with the help of the ICC.

Abbreviations: ADC, adenocarcinoma; CD56, an alias for neural cell adhesion molecule 1 (NCAM 1); CK5/14, cytokeratin 5/14; CK5/6, cytokeratin 5/6; CK7, cytokeratin 7; ICC, immunocytoology; IHC, immunohistochemistry; neg., negative; NET, neuroendocrine tumour; NSCC-NOS, non-small cell cancer-not otherwise specified; pos., positive; SQC, squamous cell carcinoma; Syn, synaptophysin; TTF-1, thyroid transcription factor 1.
### Table 3
Probabilities of different lung cancer types (showing the performance of combinations of two antibody markers)

| Cancer Type | n (a priori %) | ICC a posteriori | TTF-1+ and CK7+ | TTF-1+ and CK5/6, CK5/14- | CK5/6, CK5/14+ and p40(Δp63)+ | CK5/6, CK5/14+ and TTF-1- | CD56+ and Syn+ | TTF-1- and CK5/6, CK5/14- |
|-------------|----------------|------------------|-----------------|--------------------------|--------------------------------|---------------------------|-----------------|--------------------------|
|             |                |                  | Neg. | Pos. | Neg. | Pos. | Neg. | Pos. | Neg. | Pos. | Neg. | Pos. | Neg. | Pos. | Neg. | Pos. |
| SQC         | ICC 256 13.1   | TTF-1+ and CK7+  | 14.9 (70) | 0.1 (1) | 25.8 (51) | 1.8 (3) | 2.7 (2) | 95.0 (19) | 5.5 (22) | 89.0 (129) | – | – | 41.1 (131) | 5.4 (8) |
|             | IHC 69 15.8    | TTF-1+ and CK5/6, CK5/14- | 10.7 (3) | – | 44.2 (23) | – | 4.3 (2) | 48.0 (24) | 1.9 (1) | 64.7 (22) | – | – | 27.8 (22) | 11.1 (1) |
| ADC         | ICC 972 49.6   | TTF-1+ and CK5/6, CK5/14- | 27.5 (129) | 96.6 (658) | 7.6 (15) | 91.0 (152) | 49.3 (36) | 5.0 (1) | 52.4 (209) | 2.8 (4) | 9.5 (2) | 11.8 (4) | 51.7 (165) | 6.7 (10) |
|             | IHC 258 59.2   | TTF-1+ and CK5/6, CK5/14- | 53.6 (15) | 97.3 (36) | 26.9 (14) | 94.4 (34) | 80.4 (37) | 24.0 (12) | 81.5 (44) | 11.8 (4) | 55.6 (5) | 18.8 (3) | 57.0 (45) | 33.3 (3) |
| NSCC- NOS   | ICC 464 23.7   | TTF-1+ and CK5/6, CK5/14- | 54.6 (256) | 2.2 (15) | 65.7 (130) | 6.0 (10) | 46.6 (34) | – | 40.4 (161) | 7.6 (11) | 38.1 (8) | 2.9 (1) | 6.0 (19) | 86.6 (129) |
|             | IHC 60 13.8    | TTF-1+ and CK5/6, CK5/14- | 32.1 (9) | 2.7 (1) | 26.9 (14) | 5.6 (2) | 15.2 (7) | 26.0 (13) | 16.7 (9) | 20.6 (7) | 44.0 (4) | 6.3 (1) | 13.9 (11) | 55.6 (5) |
| NET         | ICC 268 13.7   | TTF-1+ and CK5/6, CK5/14- | 3.0 (14) | 1.0 (7) | 1.0 (2) | 1.2 (2) | 1.4 (1) | – | 1.8 (7) | 0.7 (1) | 52.4 (11) | 85.3 (29) | 1.3 (4) | 1.3 (2) |
|             | IHC 49 11.2    | TTF-1+ and CK5/6, CK5/14- | 3.6 (1) | – | 1.9 (1) | – | 2.0 (1) | – | 2.9 (1) | – | 75.0 (12) | – | 1.3 (1) | – |
| Total       | ICC 1960 100.0 | TTF-1+ and CK5/6, CK5/14- | 469 | 681 | 198 | 167 | 73 | 20 | 399 | 145 | 21 | 34 | 319 | 149 |
|             | IHC 436 100.0  | TTF-1+ and CK5/6, CK5/14- | 28 | 37 | 52 | 36 | 46 | 50 | 54 | 34 | 9 | 16 | 79 | 9 |

**Note:** The a priori probability shows computation of existing data before performing an investigation. The a posteriori probability is for the diagnosis with the help of ICC.

**Abbreviations:** ADC, adenocarcinoma; CD56, an alias for neural cell adhesion molecule 1 (NCAM 1); CK5/14, cytokeratin 5/14; CK5/6, cytokeratin 5/6; CK7, cytokeratin 7; ICC, immunocytology; IHC, immunohistochemistry; neg., negative; NET, neuroendocrine tumour; NSCC-NOS, non-small cell cancer—not otherwise specified; pos., positive; SQC, squamous cell carcinoma; Syn, synaptophysin; TTF-1, thyroid transcription factor 1.
The use of TTF-1, CK7, CK5/6–CK5/14, or p40 (∆Np63), respectively, vs 86.6% for a combination of TTF-1 and CK5/6–CK5/14, P < 0.05; Tables 2 and 3).

The use of combined antibodies does not really improve the a posteriori probability for the group of NET subtypes: 90.3% and 87.5% for the isolated use of CD56 and Syn, respectively, vs 85.3% for CD56 and Syn in combination, P > 0.5; Tables 2 and 3).

### 3.4 Comparison of routine vs immunocytoLOGY-based diagnoses

Comparisons of the results of lung cancer typing based on the standard Giemsa staining alone, with the diagnoses following ICC globally and as finalised on a clinical or cytological versus a histological basis, are presented as cross matrices in Tables 5-7.

The highest agreement between Giemsa and all ICC-based cytological diagnoses were observed for ADC (781 of 972 cases, 89.2%) and NET (239 of 268, 80.3%), whereas the proportions correctly diagnosed cases based on ICC were lower for SQC (180 of 256, 70.3%) and NSCC (295 of 464; 63.6%, Table 5). Comparisons made when dividing the patients between clinically/cytologically versus histologically are said to be TTF-1 negative.6,10 Consistent with these earlier findings, we found TTF-1 negativity in 13.0% (122/935) of ADC, 90.5% (143 of 158) of SQC, and 22.8% (55/241) of NET specimens (Table 2).

In the case of SQC, however, such a procedure does not lead to any significant improvement in the a posteriori probabilities (for ICC, 87.3% vs 95.0% or 89.0%; for IHC, 49.0% vs 48.0% or 64.7%; Tables 2 and 3). As expected, ICC is of greatest importance for the diagnosis of NSCC-NOS. If there is no expression of TTF-1 and CK5/6–CK5/14 antibodies, the a posteriori probability of the presence of NSCC-NOS increases significantly to 86.6% for the combined antibody use, compared to the range of 35.5% to 52.5% for the use of either p40 (∆Np63), CK5/6–CK5/14, CK7, or TTF-1 alone (Tables 2 and 3).

In addition to positive reactions, knowledge of negative results is also important for determining the limitations of individual antibodies in immunostaining.10

The sensitivity and specificity of individual antibodies have been well studied in the literature.7,10 In contrast to assessing the predictive power of factors (antibodies) for an underlying tumour type individually, in practice we have to calculate the predictive power of factors, possibly including morphological and clinical factors, in combinations. Bayes’ theorem is in this regard a well suited, flexible statistical and analytical approach, and it allows for information independent from the size of the individual groups.18

It is well known that lung cancers frequently show histological heterogeneity.72,22 The heterogeneity is particularly important with regard to the gap in size between the sample and the tumour that is a methodological outcome of using small biopsies and cytology specimens. Therefore both lung cancer typing using standard stains and assessing immunostaining are a challenge.

In the absence of glandular differentiation, or a squamous epithelial or neuroendocrine growth pattern, cytological assessment of the tumour type present using standard stains must fail.6,24 As a consequence of the limited sensitivity and specificity values of the individual antibodies, both negative and positive ICC stains always require differential diagnoses of multiple classifications in the clinical-morphological context. Appropriate internal positive controls are a prerequisite for a correct negative evaluation.75

For TTF-1, it is generally believed that not all lung cancers express TTF-1.10,26,27 About 20% to 25% of pulmonary ADC, 11% of SCLC, up to 96% of SQC, 0% to 100% of TC/AC, and 69% of LCNEC are said to be TTF-1 negative.1,10 Consistent with these earlier findings, we found TTF-1 negativity in 13.0% (122/935) of ADC, 90.5% (143 of 158) of SQC, and 22.8% (55/241) of NET specimens (Table 2).

Kimbrell et al7 showed that between 5% and 77% of SQCs may show CK7 expression, albeit weaker than ADC. Most adenosquamous carcinomas, large cell carcinomas, and pleomorphic carcinomas as well as LCNEC are also said to express CK7.10,23,28 Besides, for distinguishing between sarcomatoid lung carcinoma and sarcomatoid mesothelioma, GATA binding protein3 (GATA3) is recommended with its 100% sensitivity, usually being positive in the latter.29

In the present ICC examination, 91.3% (735/805) of the ADC, 20.5% (15/73) of the SQC, about half (138/274) of all NSCC, and around one third (7/222) of all NET assays were CK7-positive (Table 2).

Only focal or weak p40 (∆Np63) colour reactions can occur in both ADC and other tumors.30,31 Accordingly, a cut-off rate of more than 50% of stained tumour nuclei has been proposed as defining a positive reaction. Regardless of this, superficial parts of SQC associated with cornification are often p40 (∆Np63)-negative.10,31 If one assumes that these superficial parts of an SQC may be preferably sampled when tissue is removed for cytology, then the comparatively low proportion of p40 (∆Np63)-expressing tumours could be explained in the present analysis.32-34 In our experience (manual ICC),

---

**Discussions:**

The isolated use of one antibody (TTF-1, CK7, CK5/6–CK5/14, or p40 (∆Np63)) in ICC increases the probability of recognising ADC from 49.6% a priori to 77.7% (TTF-1) or 82.1% (CK7) a posteriori, or similarly for SQC, from 13.1% a priori to 87.3% a posteriori (Table 2). In the case of NET, comparable increases in probability can be observed with CD56 or Syn (13.7% a priori vs 90.3% and 87.5% a posteriori, respectively; Table 2). In the majority of cases, however, the morphological diagnosis of NET can be achieved with a high degree of certainty based on routine staining (80.3%, Table 7).

Automated IHC staining systems consume one slide per antibody. Multiple, manual staining on a single slide is an essential prerequisite for a correct negative evaluation.16 As a consequence of the limited sensitivity and specificity values of the individual antibodies, both negative and positive ICC stains always require differential diagnoses of multiple classifications in the clinical-morphological context. Appropriate internal positive controls are a prerequisite for a correct negative evaluation.75

For TTF-1, it is generally believed that not all lung cancers express TTF-1.10,26,27 About 20% to 25% of pulmonary ADC, 11% of SCLC, up to 96% of SQC, 0% to 100% of TC/AC, and 69% of LCNEC are said to be TTF-1 negative.1,10 Consistent with these earlier findings, we found TTF-1 negativity in 13.0% (122/935) of ADC, 90.5% (143 of 158) of SQC, and 22.8% (55/241) of NET specimens (Table 2).

Kimbrell et al7 showed that between 5% and 77% of SQCs may show CK7 expression, albeit weaker than ADC. Most adenosquamous carcinomas, large cell carcinomas, and pleomorphic carcinomas as well as LCNEC are also said to express CK7.10,23,28 Besides, for distinguishing between sarcomatoid lung carcinoma and sarcomatoid mesothelioma, GATA binding protein3 (GATA3) is recommended with its 100% sensitivity, usually being positive in the latter.29

In the present ICC examination, 91.3% (735/805) of the ADC, 20.5% (15/73) of the SQC, about half (138/274) of all NSCC, and around one third (7/222) of all NET assays were CK7-positive (Table 2).

Only focal or weak p40 (∆Np63) colour reactions can occur in both ADC and other tumors.30,31 Accordingly, a cut-off rate of more than 50% of stained tumour nuclei has been proposed as defining a positive reaction. Regardless of this, superficial parts of SQC associated with cornification are often p40 (∆Np63)-negative.10,31 If one assumes that these superficial parts of an SQC may be preferably sampled when tissue is removed for cytology, then the comparatively low proportion of p40 (∆Np63)-expressing tumours could be explained in the present analysis.32-34 In our experience (manual ICC),
ELMAS ET AL.

CK5/14 (with pretreatment in solution at pH 6.0) which was selected for technical reasons, offers a good alternative to p40 (ΔNp63) and CK5/6 in order to detect tumours with squamous differentiation.

It is very important to know the limitations of ICC for lung cancer subclassification or rare tumour types such as LCNEC, basaloid squamous cell carcinoma, or NSCC with NET differentiation.\(^5,6,8,35\) In order to compensate for the advantages and disadvantages of the individual antibodies, antibody combinations are used in practice.\(^{10,29,36,37}\) The current WHO classification for lung tumours recommends the use of neuroendocrine markers only in the presence of neuroendocrine growth patterns (organoid cell nests, rosette-like structures, palisade-like patterns, etc.).

| TABLE 4 | Probabilities of different lung cancer types (showing the performance of using combinations of more than two antibody markers) |
|----------|----------------------------------------------------------------------------------------------------------------------------------|
|          | ICC a posteriori probability % (n)                                                                                                                                                 |
|          | TTF−1+ and CK7+ and CK5/6, CK5/14− | TTF−1− and CK7− and CK5/6, CK5/14+ | TTF−1− and CK7− and CK5/6, CK5/14+ and p40(ΔNp63)+ |
|          | Neg. | Pos. | Neg. | Pos. | Neg. | Pos. |
| SQC      | 256  | 13.1 | 34.3 (61) | – | 1.4 (3) | 89.2 (58) | 5.5 (22) | 89.0 (129) |
| ADC      | 972  | 49.6 | 10.7 (19) | 97.0 (98) | 53.7 (115) | 3.1 (2) | 52.4 (209) | 2.8 (4) |
| NSCC-NOS | 464  | 23.7 | 54.5 (97) | 3.0 (3) | 44.4 (95) | 7.7 (5) | 40.4 (161) | 7.6 (11) |
| NET      | 268  | 13.7 | 0.6 (1) | – | 0.5 (1) | – | 1.8 (7) | 0.7 (1) |
| Total    | 1960 | 100.0 | 178 | 101 | 214 | 65 | 399 | 145 |

Note: The a priori probability shows computation of existing data before performing an investigation. The a posteriori probability is for the diagnosis with the help of ICC.

Abbreviations: ADC, adenocarcinoma; CD56, an alias for neural cell adhesion molecule 1 (NCAM 1); CK5/14, cytokeratin 5/14; CK5/6, cytokeratin 5/6; CK7, cytokeratin 7; ICC, immunocytology; neg., negative; NET, neuroendocrine tumour; NSCC-NOS, non-small cell cancer—not otherwise specified; pos., positive; SQC, squamous cell carcinoma; Syn, synaptophysin; TTF-1, thyroid transcription factor 1.

| TABLE 5 | Lung cancer diagnoses and subtyping for Giemsa staining alone vs with immunocytochemistry (final diagnosis on either clinical/cytological or histological basis, n = 1960) |
|----------|----------------------------------------------------------------------------------------------------------------------------------|
|          | Final diagnosis based on biopsy/ICC (n)                                                                                           | Lung cancer typing (based on Giemsa staining only)                                                                 |
|          | SQC | ADC | NSCC-NOS | NET | Other |
| SQC      | 256 | 180 | 11 | 61 | 0 | 4 |
| ADC      | 972 | 781 | 32 | 295 | 11 | 14 |
| NSCC-NOS | 464 | 229 | 906 | 516 | 253 | 56 |
| NET      | 268 | 1 | 5 | 2 | 239 | 21 |
| Total    | 1960 | 92 | 349 | 253 | 135 | 29 |

Note: Bold digits display the final diagnosis confirmed by ICC.

Abbreviations: ADC, adenocarcinoma; ICC, immunocytochemistry; n, number; NET, neuroendocrine tumour; NSCC-NOS, non-small cell cancer—not otherwise specified; SQC, squamous cell carcinoma.

| TABLE 6 | Lung cancer diagnoses and subtyping for Giemsa staining alone vs with immunocytochemistry (final diagnosis on clinical/cytological basis, n = 858) |
|----------|----------------------------------------------------------------------------------------------------------------------------------|
|          | Final diagnosis based on biopsy/ICC (n)                                                                                           | Lung cancer typing (based on Giemsa staining only)                                                                 |
|          | SQC | ADC | NSCC-NOS | NET | Other |
| SQC      | 95  | 69  | 5 | 20 | 0 | 1 |
| ADC      | 383 | 8   | 291 | 76 | 0 | 8 |
| NSCC-NOS | 235 | 14  | 53  | 155 | 5 | 8 |
| NET      | 145 | 1   | 0   | 2  | 130 | 12 |
| Total    | 858 | 92  | 349 | 253 | 135 | 29 |

Note: Bold digits display the final diagnosis confirmed by ICC.

Abbreviations: ADC, adenocarcinoma; ICC, immunocytochemistry; n, number; NET, neuroendocrine tumour; NSCC-NOS, non-small cell cancer—not otherwise specified; SQC, squamous cell carcinoma.
4.0% or 10.0%) of the actual SQC, ADC, or NSCC samples expressed CD56 or Syn. In contrast, the vast majority of NETs are CD56- or Syn-positive (90.3% and 87.5%, respectively).

In the literature only sufficiently large series have the necessary statistical power for assessing the differential diagnostic value of different antibody combinations.\textsuperscript{5,6,10,35,38,39} In accordance with the WHO 2015 classification and several earlier studies, this study shows that a combination of two antibodies, eg two positive or one positive and one negative, can lead to a significant improvement in subtyping only for ADC (an increase from 77.7% to 96.6% or 91%; Table 3).\textsuperscript{5,6,10,35,38,39}

However, this does not apply to the diagnosis of SQC. If the standard stains do not show characteristic growth patterns for ADC, SQC, or SCLC, and if the tumours also do not show antibody staining typical for ADC or SQC, it is generally believed to be NSCC-NOS.\textsuperscript{6,10} The diagnosis of NSCC-NOS, like the detection of ADC, can be improved through the combined use of two antibodies. The a posteriori probability of NSCC-NOS increases to 86.6% in the absence of TTF-1 and CK5/6–CK5/14 expression, compared to the range of 35.5%–52.5% for the use of p40 (ΔNp63), CK5/6–CK5/14, CK7, or TTF-1 alone (Tables 2 and 3). With such a procedure, the proportion of NSCC-NOS was reduced from 513 cases in samples with standard Giemsa staining to only 464 cases after ICC (Table 5). Additionally, the effect of using three different antibodies combined was analysed, yielding no significant increase in the a posteriori probabilities: to 97.0% for ADC with TTF-1+, CK7+, and CK5/6–CK5/14–; to 89.2% for SQC with TTF-1+, CK7–, and CK5/6–CK5/14+ (Table 4).

Although a majority of lung carcinomas can be correctly typed in cytological samples using the standard Giemsa stain, ICC appears to be suitable to improve the reliability of cytopathological diagnostics when compared either globally (an increase of 465 diagnoses in 1960 cases), or when analysing only diagnoses confirmed either clinically/cytologically (230 of 867) or histologically (286 of 1118) as well (Tables 5-7).

Based on morphological and clinical data, the use of two antibodies appears to be sufficient for the reliable detection of primary lung cancer types. This applies both to final diagnoses that are confirmed clinically/cytologically only and those that are histologically confirmed. Laboratories using validated ICC on smears and/or cytopspins should feel reassured that this is a reliable and well-accepted technique.

Additionally, the use of multiple antibodies on one slide guarantees resource-efficient processing and maximises the amount of tissue available for molecular testing.

Beyond the calculation of sensitivity and specificity of individual factors, the Bayesian method allows ranking the importance of different influencing factors for the diagnostic process. Against the background of an increasingly complex practice, the Bayesian approach could lead to a better interpretation of the analytic results for further translational analysis in the future; however more studies are needed to investigate the performance of the method.

**ACKNOWLEDGMENT**
We wish to thank the following individual for critical reading: Prof Dr Martin Reck.

**CONFLICT OF INTEREST**
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**AUTHOR CONTRIBUTIONS**
H. Elmas: Conceptualisation, data curation, statistical analysis, formal analysis, writing—original draft, writing—review and editing. R. Diel: Statistical analysis, data curation, investigation, methodology, formal analysis, writing—editing. B. Önal: Conceptualisation, investigation, resources, writing—review and editing. G. Sauter: Writing—review and editing, F. Stellmacher: Formal analysis, L. Welker: Conceptualisation, project administration, statistical analysis, resources, supervision, writing and/or revising the manuscript.

**DATA AVAILABILITY STATEMENT**
The data that support the findings of this study are available from the corresponding author upon reasonable request.
REFERENCES

1. Barta JA, Powell CA, Wisnivesky JP. Global epidemiology of lung cancer. Ann Glob Health. 2019;85(1):8.
2. Ross LG, Melamed MR. Tumors of the lung: conventional cytology and aspiration biopsy, Melamed MR eds. Diagnostic Cytology and its Histopathological Basis, 5th edn.: Lippincott, Williams & Wilkin; 2006:643-712.
3. Günel Ö, Önal B, Par O, Karakaş S. Diagnostic value of cytopathology in lung cancer-3243 sampling, 1333 patients. Turkish J Pathol. 1995;11(2):319-323 (in Turkish).
4. Önal B, Erozan Y. Sitopatolojinin yolculuğu. In: Önal B, ed. Sitopatoloji. Quintessence Publishing; 2016:1-4 (in Turkish).
5. Travis WD, Brambilla E, Burke AP, Marx A, Nicholson AG eds. Tumor of the lung. WHO Classification of Tumours of the Lung, Pleura, Thymus and Heart, 4th edn, IARC Press; 2015:1-150.
6. Travis WD. Lung cancer pathology: current concepts. Clin Chest Med. 2020;41(1):67-85.
7. Wessels S, Muley T, Christopoulos P, et al. Comprehensive serial biobanking in advanced NSCLC: feasibility, challenges and perspectives. Transl Lung Cancer Res. 2020;9(4):1000-1014.
8. Sung S, Shirazi M, Shirazi M, Crapanzano JP, Saqi A. Pulmonary small cell carcinoma: review, common and uncommon differentials, genomics and management. Diagn Cytopathol. 2020;48(8):790-803.
9. Biancosino C, Krüger M, Vollmer L, Welker L. Intraoperative fine needle aspirations – diagnosis and typing of lung cancer in small biopsies: challenges and limitations. Diagn Pathol. 2016;11(1):59.
10. Yatabe Y, Dacic S, Borczuk AC, et al. Best practices recommendations for diagnostic immunohistochemistry in lung cancer. J Thorac Oncol. 2019;14(3):377-407.
11. Roy-Chowdhuri S, Dacic S, Ghofrani M, et al. Collection and handling of thoracic small biopsy and cytology specimens for ancillary studies: guideline from the College of American Pathologists in collaboration with the American College of Chest Physicians, Association for Molecular Pathology, American Thoracic Society, Pulmonary Pathology Society, Papanicolaou Society of Cytopathology, Society of Interventional Radiology. Arch Pathol Lab Med. 2021;144(8):933-958.
12. Satturwar S, Rekhtman N, Lin O, Pantanowitz L. An update on touch preparations of small biopsies. J Am Soc Cytopathol. 2020;9(5):322-331.
13. Elmas H, Lammers M, Welker L. Immunocytochemical diagnosis: selection and technical application of proper antibodies. In: Önal B, ed. Cytopathology. Türkiyə Kliniki: 2018:94-104 (in turkish).
14. Dong Y, Li Y, Liu R, et al. Secretagigin, a marker for neuroendocrine cells, is more sensitive and specific in large cell neuroendocrine carcinoma compared with the markers CD56, CgA, Syn and Napsin A. Oncol Lett. 2020;20:2223-2230.
15. Baine MK, Rekhtman N. Multiple faces of pulmonary large cell neuroendocrine carcinoma: update with a focus on practical approach to diagnosis. Transl Lung Cancer Res. 2020;9(3):860-878.
16. Elmas H, Schaleschak J, Önal B. Immunocytochemistry. In: Önal B, ed. Techniques in Cytopathology from Basic to Molecular. Nobel Tıp Kitabevi: 2019:116-126 (in turkish).
17. Gustafson P. Measurement Error and Misclassification in Statistics and Epidemiology: Impacts and Bayesian Adjustments. CRC Press; 2003.
18. Vollmer RT. Differential diagnosis in immunohistochemistry with Bayes theorem. Am J Clin Pathol. 2009;131(5):723-730.
19. Kaufmann O, Fietze E, Dietel M. Immunohistochemische Diagnostik bei Karzinommetastasen mit unbekanntem Primärtumor. Pathologie. 2002;23(3):183-197.
20. Welker L, Jörres RA, Costabel U, Magnussen H. Predictive value of BAL cell differentials in the diagnosis of interstitial lung diseases. Eur Respir J. 2004;24(6):1000-1006.
21. Bruckmann NM, Rischpler C, Kirchner J, et al. Correlation between contrast enhancement, standardized uptake value (SUV), and diffusion restriction (ADC) with tumor grading in patients with therapy-naive neuroendocrine neoplasms using hybrid 68Ga-DOTATOC PET/MRI. Eur J Radiol. 2021:137:109588.
22. Travis WD, Dacic S, Wistuba I, et al. IASLC multidisciplinary recommendations for pathologic assessment of lung cancer resection sections after neoadjuvant therapy. J Thorac Oncol. 2020;15(5):709-740.
23. Scaglotti G, Brodowicz T, Shepherd FA, et al. Treatment-by-histology interaction analyses in three phase III trials show superiority of pemetrexed in nonsquamous non-small cell lung cancer. J Thorac Oncol. 2011;6(1):64-70.
24. Compérat E, Zhang F, Perrotin C, et al. Variable sensitivity and specificity of TTF-1 antibodies in lung metastatic adenocarcinoma of colorectal origin. Mod Pathol. 2005;18(10):1371-1376.
25. Ordóñez NG. Value of thyroid transcription factor-1 immunostaining in tumor diagnosis: a review and update. Appl Immunohistochem Mol Morphol. 2012;20(5):429-444.
26. Prabhakaran S, Woo WLY, Xing G, et al. The incidence of labeling of non-lung adenocarcinomas with antibodies against TTF-1 and diagnostic implications. Appl Immunohistochem Mol Morphol. 2020;28(6):471-476.
27. Kimbrell HZ, Gustafson KS, Huang M, Ehya H. Subclassification of non-small cell lung cancer by cytologic sampling: a logical approach with selective use of immunocytochemistry. Acta Cytol. 2012;56(4):419-424.
28. Rossi G, Mengoli MC, Cavazza A, et al. Large cell carcinoma of the lung: clinically oriented classification integrating immunohistochemistry and molecular biology. Virchows Arch. 2014;464(1):61-68.
29. Michael C, Hiroshima K, Hjerpe A, et al. Malignant-primary (MAL-P) (Mesothelioma). In: Chandra A, Crothers B, Kurtycz D, Schmitt F, eds. The International System for Serous Fluid Cytopathology: Springer; 2020;63:98. 10.1007/978-3-030-53908-5_6
30. Cabibi D, Bellavia S, Giannone AG, et al. TTF-1/p63-positive poorly differentiated NSCLC: a histogenetic hypothesis from the basal reserve cell of the terminal respiratory unit. Diagnostics. 2020;10(1):25.
31. Rekhtman N, Paik PK, Arcila ME, et al. Clarifying the spectrum of driver oncogene mutations in biomarker-verified squamous carcinoma of lung: lack of EGFR/KRAS and presence of PIK3CA/AKT1 mutations. Clin Cancer Res. 2012;18(4):1167-1176.
32. Sailer V, Lüders C, Cahn W, Pelzer V, Kristiansen G. Immunostaining of ΔNp63 (using the p40 antibody) is equal to that of p63 and CK5/6 in high-grade ductal carcinoma in situ of the breast. Virchows Arch. 2015;467(1):67-70.
33. Pelosi G, Rossi G, Cavazza A, et al. ΔNp63 (p40) distribution inside lung cancer: a driver biomarker approach to tumor characterization. Int J Surg Pathol. 2013;21(3):229-239.
34. Rekhtman N, Ang DC, Sima CS, Travis WD, Moreira AL. Immunohistochemical algorithm for differentiation of lung adenocarcinoma and squamous cell carcinoma based on large series of whole-tissue sections with validation in small specimens. Mod Pathol. 2011;24(10):1348-1359.
35. Da Cruz V, Yvorel V, Castelló F, et al. Histopathological subtyping is a prognostic factor in stage IV lung adenocarcinoma. Lung Cancer. 2020;147:77-82.
36. Ao MH, Zhang H, Sakowski L, et al. The utility of a novel triple marker (combination of TTF1, napsin A, and p40) in the subclassification of non-small cell lung cancer. Hum Pathol. 2014;45(5):926-934.
37. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature*. 2001;414(6859):105-111.

38. Whithaus K, Fukuoka J, Prihoda TJ, Jagirdar J. Evaluation of napsin A, cytokeratin 5/6, p63, and thyroid transcription factor 1 in adenocarcinoma versus squamous cell carcinoma of the lung. *Arch Pathol Lab Med*. 2012;136(2):155-162.

39. Yu H, Li L, Liu D, Li WM. Expression of TTF-1, NapsinA, P63, CK5/6 in lung cancer and its diagnostic values for histological classification. *Journal of Sichuan University (Medical Science Edition)*. 2017;48(3):336-341 (in chinese).

How to cite this article: Elmas H, Diel R, Önal B, Sauter G, Stellmacher F, Welker L. Recommendations for immunocytochemistry in lung cancer typing: An update on a resource-efficient approach with large-scale comparative Bayesian analysis. *Cytopathology*. 2022;33:65–76. https://doi.org/10.1111/cyt.13051