Subtyping of Undifferentiated Non-small Cell Carcinomas in Bronchial Biopsy Specimens

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Introduction: The emergence of treatments for non-small cell lung carcinoma (NSCLC) with differential efficacy and toxicity between subtypes has highlighted the importance of specific pathologic NSCLC subtyping. Most NSCLCs are inoperable, and pathologic diagnosis is made only on small tissue samples that are prone to diagnostic inaccuracy. In a substantial proportion of cases, standard morphology cannot specifically subtype the tumor, necessitating a diagnosis of NSCLC-not otherwise specified (NOS). Histochemical staining for mucin and immunohistochemical (IHC) identification of NSCLC subtype-associated markers could help predict the final subtype of resected NSCLCs diagnosed as NSCLC-NOS on preoperative bronchial biopsy samples.

Methods: Paraffin sections of 44 bronchial biopsy samples diagnosed as NSCLC-NOS were stained for mucin (Alcian blue/periodic acid Schiff) and thyroid transcription factor 1 by IHC–(markers of adenocarcinoma), and for S100A7, cytokeratin 5/6, high molecular weight cytokeratins, and p63 proteins–markers of squamous cell carcinoma. A predictive staining panel was derived from statistical analysis after comparing staining profiles with the final postsurgical NSCLC subtype. This panel was prospectively applied to 82 small biopsy samples containing NSCLC.

Results: True NSCLC subtype of undifferentiated NSCLC samples was best predicted using Alcian blue/periodic acid Schiff plus p63 and thyroid transcription factor 1 IHC, allowing specific subtyping in 73% of NSCLC-NOS cases with 86% accuracy. When applied prospectively, this staining panel showed 100% concordance with specific NSCLC morphologic subtyping in small biopsies.

Conclusion: This approach can facilitate treatment selection by accurately predicting the subtype in undifferentiated NSCLC biopsies, reducing to 7% the proportion of cases without a definite or probable histologic subtype.

Until recently, the histologic subtyping of non-small cell lung carcinoma (NSCLC) was not clinically or therapeutically important, because of a lack of differential treatment options for NSCLC subtypes. The relevant distinction that determine treatment choice had always been between small cell and NSCLC, a differential diagnosis that pathologists are able to make consistently and accurately.[1–3] The emergence of treatments with differential activity (e.g., pemetrexed) or limited indication (e.g., bevacizumab) in subtypes of NSCLC has placed a new emphasis on the importance of accurate subtyping.[4,5]

In practice, resection rates for lung cancer are ~10 to 15%. Thus, relatively few cases undergo complete histologic examination, and the subsequent application of the World Health Organization (WHO) classification of primary lung carcinoma is limited.[6] In consequence, the majority of patients are managed on the basis of a diagnosis made on a single small tumor biopsy or even a cytologic sample. Many NSCLC are heterogeneous, although not in terms of mixed differentiation, they certainly show areas of undifferentiated tumor that lack the light-microscopic features of differentiation visible in other parts of the lesion. This combination of small diagnostic sample size and tumor heterogeneity results in many diagnostic samples being unrepresentative of the true tumor histology and explains the consequent reported lack of consistency and accuracy in subtyping NSCLCs in this context.[7–12] In addition, this is also why large cell carcinoma, as defined in the WHO classification, cannot be accurately diagnosed in small biopsy or cytology material. Reported figures for diagnostic accuracy of large cell carcinoma are ~50% or less, whereas for either squamous cell or adenocarcinoma, it is higher, ranging from 65 to 90%. When undifferentiated NSCLC is present in such samples, a diagnosis of non-small cell carcinoma, not otherwise specified (NSCLC-NOS) is recommended.[8,13] Some studies have found that this diagnosis accounts for ~25% of non-small cell carcinoma diagnoses in small biopsy samples and 45% of cytology samples.[9]

When used appropriately, immunohistochemical (IHC) staining can allow tumor subtype to be inferred when histologic morphology is nonspecific. Antibodies to cytokeratins 5/6 (CK5/6) and high molecular weight cytokeratins (HM-WCK [CK 1, 5, 8, and 14]-34βE12) and to S100A7 and p63 proteins have been used to infer squamous phenotypes.[14–24] Mucin is a useful diagnostic feature of many adenocarcinomas and 70 to 85% of lung adenocarcinomas express thyroid transcription factor (TTF) 1.[25–27] In this study, we evaluate the ability of these IHC markers and mucin staining to predict the likely NSCLC subtype in cases where the bronchial biopsy diagnosis was NSCLC-NOS and the tumor was subsequently resected and definitively categorized.

MATERIALS AND METHODS

Fifty cases of surgically resected lung cancer, preoperatively diagnosed as NSCLC-NOS on bronchial biopsy samples, were selected from the case files of the Department of Pathology at Aberdeen Royal Infirmary. Final diagnoses and histologic NSCLC subtypes were recorded from the departmental case records for each subsequent surgical resection specimen, with resort to slide review where necessary (Table 1). All diagnostic materials (slides and tissue blocks) were readily available. Four cases had insufficient tissue remaining in the original bronchial biopsy paraffin-embedded tissue block to allow inclusion in this study.

Forty-six cases had 4-μm thick sections cut and mounted on charged Chem Mate slides (Dako, Ely, UK). Sections from each case were stained for mucin (Alcian blue/periodic acid Schiff [AB/PAS]-without nuclear counterstain) and with a panel of antibodies against S100A7 (1:100; Novocastra, Peterborough, UK), p63 (1:50; Novocastra), CK 5/6 (1:200; Zymed, Paisley, UK), 34βE12-HMWCK (1:100; Dako), and TTF1 (1:50; Dako) using standard immunohistochemistry methodology (heat antigen retrieval, DAKO Benchmark XT autostainer, and Ultraview visualization [Dako]). Of the 46 cases, two further cases were excluded as the relevant tumor tissue cut out on further sectioning; 44 cases had all immunostains and mucin histochemistry performed.

Any intracytoplasmic mucin droplet staining with AB/PAS in tumor cells was considered positive. The IHC slides were double scored by two of the authors (P.S.L. and K.M.K.). Intensity of staining was graded into four semiquantitative categories: (a) absent = score 0, (b) light = score 1, (c) intermediate = score 2, and (d) strong = score 3. Similarly, the proportion of cells staining was categorized as follows: (a) none = score 0, (b) 1 to 10% = score 1, (c) 10 to 50% = score 2, and (d) >50% = score 3.

Neural nets were used to optimize the categorization of IHC scores into positive or negative, to provide the best predictive cutoff values for the semiquantitative scores obtained from each antibody.[28] The raw scores for each marker for each biopsy specimen were fed into simple neural nets comprising three nonlinear perceptrons (neurones), arranged into two layers. Given the limited data set, the nets were trained using all of the scored biopsy specimens. Analysis of net outputs for each of the observed staining patterns for each marker divided those patterns into areas that were nonpredictive (negative) or predictive (positive) for each tumor subtype. In transition areas between nonpredictive and predictive staining patterns, where no input scores were available from our dataset, the output was recorded as no data. Positive and negative predictive values, sensitivity, and specificity were calculated for each marker and for various combinations of markers to predict tumor histotype.

After establishing a set of predictive tests, based on the scoring system described above and the predictive score thresholds subsequently defined by the retrospective series of 44 cases, we applied this diagnostic panel of combined AB/PAS plus TTF1, p63, and CK5/6 IHC prospectively in 82 small biopsy samples of lung cancer from various sites (Table 2). Forty-one of these cases were specifically classifiable on the hematoxylin and eosin (HE) stain as either squamous cell or adenocarcinoma—Table 2; six tumors were subsequently resected. Of the remaining 41 cases, each were considered NSCLC-NOS on the HE stained sections. Only two of these cases were resected and definitively histotyped. This work was performed with the appropriate ethical approval from Grampian research ethics committee.

TABLE 1. Breakdown of Final Resection and IHC-Predicted Subtypes of 44 Resected Lung Carcinomas

| Resection Histology | N | IHC Predicted Phenotype | | |
		Squamous	Adenocarcinoma	Null
Squamous cell carcinoma	23[a]	22	0	1
Adenocarcinoma	11[b]	0	8	3
Large cell carcinoma	8[c]	4	1	3
Adenosquamous carcinoma	2	1	1	0

Null refers to a nonpredictive immunophenotype (see text).
[a] Two of these cases were basaloid variant, and one showed sarcomatoid carcinoma.
[b] One was combined with large cell neuroendocrine carcinoma.
[c] Two were basaloid variants.
IHC, immunohistochemical.

TABLE 2. Summary of Cases Prospectively Stained Using the Diagnostic Panel Determined by the Retrospective Analysis

Small Biopsy Type	No. Cases (Total = 82)
Bronchial biopsy	65
Transthoracic lung core biopsy	12
Liver biopsy	3
Lymph node biopsy	2

Pre Panel HE Diagnosis on Prospective Cases	No. Cases (Total = 82)	Panel Prediction
Squamous cell carcinoma	25	All squamous cell carcinoma
Adenocarcinoma	16	All adenocarcinoma
NSCLC-NOS	41	19 Squamous cell carcinoma
		11 Adenocarcinoma
		11 Not predictive[a]

[a] These cases had null immunohistochemical phenotypes. One case was predicted as both squamous and adenocarcinoma.

HE, hematoxylin and eosin stain; NSCLC-NOS, non-small cell lung carcinoma-not otherwise specified.
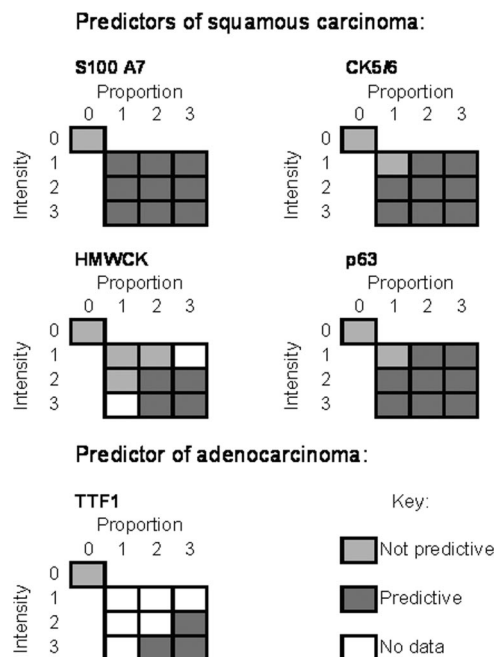
FIGURE 1. Summary diagrams of immunohistochemical staining for each of the markers. The shades indicate whether each possible combination of intensity and distribution score were considered positive (predictive of histotype) or negative (not predictive), as determined by statistical analysis (see Materials and Methods).

TABLE 3. Summary of Predictive Scores for Each Marker

Marker	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Markers to correctly predict a squamous histotype				
S100A7	36	74	64	47
p63	92	74	82	88
CK5/6	84	79	84	79
HMWK	88	74	81	82
p63 or CK5/6	92	74	82	88
Markers to correctly predict an adenocarcinoma histotype				
AB/PAS	23	100	100	76
TTF1	54	97	88	83
TTF1 or AB/PAS	69	97	90	88
Markers to correctly predict a non-squamous histotype				
p63 (−) and AB/PAS (+)	78	92	88	85

PPV, positive predictive values; NPV, negative predictive values; CK5/6, cytokeratins 5/6; HMWK, high molecular weight cytokeratins; AB/PAS, Alcian blue/periodic acid Schiff; TTF1, thyroid transcription factor 1.

RESULTS

After attrition because of inadequate biopsy material, 44 resection specimens initially diagnosed as NSCLC-NOS on small sample biopsy were included in this study. Table 1 summarizes the distribution of the final diagnosis after resection. Squamous cell carcinoma was the dominant type, accounting for just more than half of the cases; one quarter of cases were adenocarcinoma.

All of the markers showed variability in staining with respect to intensity and proportion of tumor cells stained. Overall, different tumor types showed different, but overlapping, staining profiles. Figure 1 shows the pairwise interpretation of staining scores for each marker. Any staining for S100A7 was considered predictive. Higher levels of CK5/6 and p63 staining were predictive; weak staining of <10% of the tumor cells was not predictive. In general, for TTF1, p63, CK5/6, and HMWCK, staining was either nonexistent/weak or abundant and strong. Tumors showing intermediate levels of staining were relatively less well represented in our data set. Thus, the transition areas between staining scores interpreted as negative and positive are less reliable than areas more deeply set within the negative and positive zones. Moreover, for HMWCK and TTF1, certain score combinations were not encountered and could not be extrapolated from surrounding scores by the nets. These areas were recorded as no data (Figure 1).

The ability, in numerical terms, of the four selected markers, S100A7, p63, CK5/6, and HMWCK, to correctly predict a squamous histotype are summarized in Table 3. The marker p63 showed the best sensitivity, whereas CK5/6 showed the best specificity. These scores for individual markers could not be improved by considering combination scores for more than one marker. Any combination of TTF1 and mucin positivity yielded the best figures for predicting an adenocarcinoma in the resection specimen (Table 3). To predict a nonsquamous histotype, the best results were obtained using a negative (nonpredictive—Figure 1) p63 stain and/or a positive AB/PAS stain.

Staining of preoperative samples from true large cell carcinomas (8 cases) showed a squamous profile in four cases, an adenocarcinoma profile in one case, and in three cases, a profile of low-level staining suggestive of neither squamous nor adenocarcinoma (a nonpredictive null immunophenotype; Table 1). After ignoring those cases diagnosed as adenosquamous carcinoma at resection, of 23 definitive squamous cases, 22 showed a squamous immunophenotype at biopsy and one showed a null immunophenotype. Of 11 definitive adenocarcinomas, eight showed a consistent adenocarcinoma immunophenotype on biopsy and three showed a null immunophenotype. These data mean that of the seven cases showing a null immunophenotype at biopsy, one was squamous, three were adenocarcinomas, and three were true large cell carcinoma at resection.

From the above data, a diagnostic panel of combined AB/PAS for mucin plus IHC for TTF1, p63, and CK5/6 was determined. Although the statistical data suggested that the addition of another squamous marker in addition to p63 would not improve the efficacy of prediction of NSCLC histotype, it was recognized that in cases where the p63 score falls into the transitional area between very weak and very strong staining—i.e., when scores of 1 + 2 (intensity + proportion), 2+2, or 2+1 were allocated—then the predictive value is less certain, and the ~50 addition of CK5/6 would seem prudent.

Eighty-two small biopsy samples were prospectively stained using this panel. Half of these cases could be specifically subtyped on HE histology alone into squamous cell or adenocarcinoma (Table 2). All of these cases showed a predictive staining profile entirely commensurate with the HE diagnosis. Six of these cases were subsequently resected, all of which showed the expected histotype—four squamous cell and two adenocarcinomas. Of the remaining 41 cases classified as NSCLC-NOS, the staining panel predicted squamous cell carcinoma in 19 cases, adenocarcinoma in 11 cases, leaving 11 cases with a null phenotype. Only two of these 41 NSCLC-NOS cases were subsequently resected. In one case, the squamous nature of the tumor was correctly predicted from the small biopsy using the panel. In the other case, the small sample was reported as being NSCLC-NOS after the panel, despite the p63 score being $(2 + 1)$, and, thus, predictive of a squamous phenotype. This was because the score lay in the transition area between very weak and very strong staining and the CK5/6 score was negative. Both TTF1 and mucin were negative in this second case. At resection, this latter case was definitively categorized as a poorly differentiated adenocarcinoma.

DISCUSSION

This study has focused primarily on the performance of putative markers of squamous cell (p63, CK5/6, HMWCK, and S100A7) and adenocarcinoma (mucin stain and TTF1) to predict the likely histologic subtype of a NSCLC, when applied to a biopsy sample that shows only undifferentiated NSCLC. This study is timely given the rise in prescribing licenses and treatment response and toxicity effects predicated on tumor histology.

The data presented indicated that the best positive predictive marker of squamous histology was p63 alone. However, in situations of moderate p63 staining, CK5/6 could be added. HMWCK and S100A7 were somewhat inferior as markers of squamous histotype. Combining an AB/PAS mucin stain with TTF1 IHC provided moderate sensitivity but excellent specificity for predicting an adenocarcinoma histotype.

Most of the published literature on this subject describes the expression of markers in morphologically diagnosable squamous cell or adenocarcinomas obtained from surgically resected specimens, autopsy cases, bronchial biopsy samples, or cytologic material.[16–20,23–25,29] These studies report between 75 and 100% of squamous cell carcinomas as positive for p63; most series report positive rates of >90%. Few studies provide criteria for a positive stain, although one stipulated that >10% of cells should express p63 for a case to be considered positive.[20] Zhang et al.[19] found all squamous cell carcinomas expressed HMWCK, whereas Camilo et al.[20] found only 56.2% of such tumors were positive for CK5/6. Although S100A7 has been suggested as a marker confined to squamous and large cell carcinomas,[23] our data suggest that it is neither sensitive nor specific enough to be of diagnostic value in the setting tested. Our anecdotal experience with S100A7 (data not shown) is that staining in squamous cell

carcinomas is very patchy and concentrates on well-differentiated areas, readily recognizable on the HE stained sections.

Kargi et al.[21] found that p63 and CK5/6 differentially stained easily recognizable adenocarcinomas and squamous cell carcinomas with 89% specificity and 79% sensitivity. Kaufmann et al.[14] also found that p63 and CK5/6 were superior to either marker alone in HE-diagnosable, although poorly differentiated, squamous cell carcinomas. Furthermore, they reported that if a positive result required >50% of cells to be stained, specificity rose to 99%, but sensitivity fell to 66%.

Many studies have demonstrated TTF1 expression in 70 to 85% of resected lung adenocarcinoma.[25–27,30] In the context of small biopsy or cytology samples, TTF1 stains anywhere between 60 and 92% of morphologically diagnosable adenocarcinomas. The variations are probably a function of the sample types predominant in the study[29,31–33]; more transthoracic aspiration or core samples and fewer bronchial samples will bias results toward terminal respiratory unit-type adenocarcinomas and raise the TTF1 prevalence.[26] Stainable mucin is one of the defining characteristics of pulmonary adenocarcinoma, although not all cases are positive,[34] and expression can be very patchy. There is limited published data, but it is the authors' impression that the AB/PAS stain will retain a high probability of staining central, bronchial-type, or poorly differentiated adenocarcinomas that are more likely to be TTF1 negative.[27] Kaufmann and Dietel[25] found TTF1 positivity in 75% of nonmucinous but only 10% of mucinous adenocarcinomas, hence, the value of using both TTF1 and AB/PAS in the diagnostic panel.

The studies outlined above examined morphologically diagnosable squamous cell and adenocarcinomas. Besides our own studies, there are very few other studies examining the ability of ancillary techniques to predict the true NSCLC subtype of undifferentiated biopsy samples. Rossi et al.[33] studied cases diagnosed as large cell carcinoma on bronchial biopsy and found that TTF1 or CK 7 as markers of adenocarcinoma and HMWCK as a marker of squamous cell carcinoma led to a correct prediction of NSCLC subtype in 21 of 23 resected cases. Monica et al.[35] reported the value of desmocollin-3 (DSC-3), as a squamous marker, and TTF1 in predicting the histotype of 31 cytologic samples diagnosed as NSCLC-NOS. These two markers appear to be mutually exclusively expressed in squamous and adenocarcinomas, respectively.

None of the markers is specific for a particular tumor histotype. In consequence, there is an understandable and unavoidable error rate in the predicted histotype based on the IHC staining profile. One large series of surgically resected NSCLC showed 30% of adenocarcinomas and 37% of large cell carcinomas positive for p63[16]; another found 40% of large cell carcinomas positive for TTF1.[25] TTF1 positivity has been reported in 5 to 21% of squamous cell carcinomas,[30–32] but, in our experience and that of others, TTF1 does not stain pulmonary squamous cell carcinomas.[25,27] Our data showed that although some squamous and adenocarcinomas had a null, nonpredictive immunophenotype, none showed the opposing, predictive immunophenotype when the biopsy sample was undifferentiated.

No squamous cell carcinoma showed an adenocarcinoma immunophenotype and vice versa.

Some true large cell carcinomas had an immunophenotype that suggested either adenocarcinoma or squamous cell carcinoma; an observation also made by others.[35] Ultrastructural evidence of differentiation in large cell carcinomas is also well recognized. This is commensurate with the hypothesis that many large cell carcinomas may be dedifferentiated squamous cell or adenocarcinomas, which retain their molecular but lose their morphologic differentiation.

Immunohistochemistry profiles do not feature in the diagnostic criteria for squamous cell or adenocarcinoma in the current WHO classification.[6] Marker profiles may be used as supporting evidence for a particular histotype in the absence of morphologic evidence, but they do not confirm the diagnosis. Care should be exercised in their use when reporting such cases. The authors' practice is to continue to sign out undifferentiated cases as NSCLC but add an appropriate qualification of probably squamous cell or adenocarcinoma if the IHC profile is predictive.

The treatment of advanced NSCLC has seen significant progress in recent years with the emergence of a number of agents that show differential efficacy or toxicity in certain subtypes of NSCLC.[4,5,36,37] In consequence, there is greater pressure on pathologists to be more specific in their diagnosis of small biopsy and cytology samples. A diagnosis of NSCLC-NOS should be refined where at all possible but may be unavoidable in a small proportion of cases, especially where the diagnostic sample contains very little tumor. Our results show that p63 and AB/PAS are the most efficient at predicting squamous versus nonsquamous histology, which is currently the relevant distinction to guide choice of some

newer treatments. By using a panel of markers such as suggested in this study, the predictive nature of the refined diagnosis needs to be borne in mind. There are no data to suggest that, for example, a tumor classified as NSCLC, probably squamous cell on the basis of IHC, would be resistant to pemetrexed in the same way that a morphologically diagnosable case would likely be. Those clinical trials that demonstrated differential efficacy or toxicity of an agent were based on pathologic diagnoses from a multitude of pathologists, using many sample types and variable methods to reach a diagnosis. The emergence of such drugs indicates the need for greater consideration of the accuracy and consistency of pathologic diagnosis in future clinical trials. This requires the use of standardized IHC-based histotyping and central pathologic review.

Our predictive panel can improve the diagnostic algorithm for NSCLC (Figure 2). NSCLC-NOS rates on small biopsy samples will vary according to the skill, confidence, and bias of individual pathologists. Published data suggest that ~75% of bronchial NSCLC samples may be subtyped using morphology alone.[9] Of the remaining 25% of samples, those lacking morphologic differentiation, this study shows that 73% are given a probable subtype, thus, typing is achieved in $75 + (25 \times 0.73) = 93\%$ of all NSCLC cases. The accuracy of both morphologic- and IHC-based predictive subtyping is $<100\%$ (Figure 2). The exact concordance between morphologic- and panel-predicted subtype in our prospective data suggests the use of IHC in morphologically diagnosable cases could improve this accuracy. In our NSCLC-NOS biopsy samples, inaccurately attributed cases were all accounted for by large cell carcinomas with a predictive immunophenotype. Overall, therefore, only 7% of all NSCLC bronchial biopsy samples should remain without a probable subtype after morphologic examination, IHC, and mucin staining.

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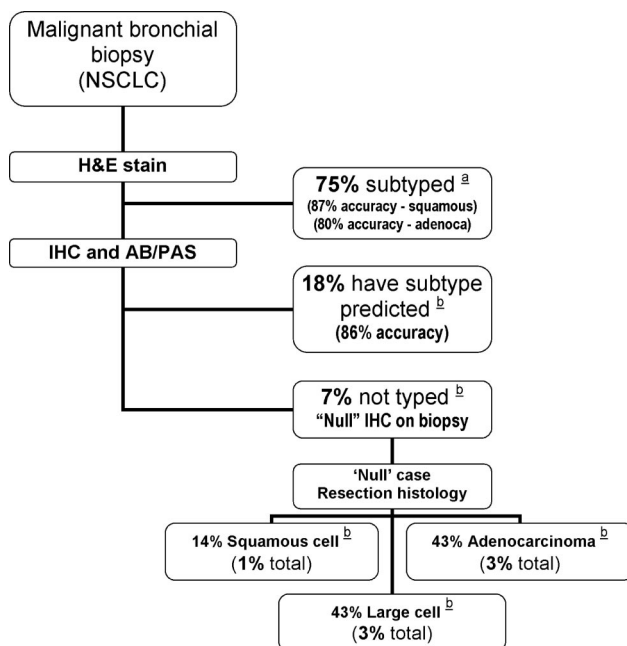
FIGURE 2. Diagnostic algorithm for classification of NSCLC in small biopsy samples. [a]Data from Edwards et al.[9] [b]All data from the current study.

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