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Original contribution

Triple marker composed of p16, CD56, and TTF1 shows higher sensitivity than INSM1 for diagnosis of pulmonary small cell carcinoma: proposal for a rational immunohistochemical algorithm for diagnosis of small cell carcinoma in small biopsy and cytology specimens☆

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Summary Pulmonary small cell carcinoma (SCLC) can be usually diagnosed based on the morphological evaluation of routine histological or cytological preparations. However, immunohistochemistry may be also necessary in problematic cases. Insulinoma-associated 1 (INSM1) has recently been reported as a highly sensitive and specific marker that displays positivity in ~90%-100% of poorly differentiated pulmonary neuroendocrine tumors. We compared diagnostic performance of INSM1 and previously reported composite marker CD56 + p16 + thyroid transcription factor-1 (TTF1) in the diagnosis of SCLC in small biopsy specimens and cytoblocks. The composite marker CD56 + p16 + TTF1 correctly classified 100% of SCLC cases, and its sensitivity was significantly higher than the sensitivity of INSM1. Among 100 SCLC cases, CD56, TTF1, and p16 each individually classified more specimens correctly than INSM1 (CD56: 84%, TTF1: 89%, p16: 95%, INSM1: 81%); the difference was statistically significant only for p16. INSM1 showed the lowest classification agreement between paired biopsy and cytoblock specimens ($\kappa = 0.182$), whereas CD56 and p16 displayed perfect agreement ($\kappa = 1$) and TTF1 showed moderate agreement ($\kappa = 0.4$). Although INSM1 is reportedly the most specific marker of SCLC, its sensitivity is not superior to p16 or composite marker CD56 + TTF1 + p16. Based on this study, we propose the following algorithm, which, in the appropriate clinical and histological context, may be useful in establishing the correct diagnosis of SCLC: First, INSM1 detection is performed, and if the result is negative, CD56 is added, followed successively

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by p16 and TTF1 if all previously applied markers are negative. This approach should detect most, if not all, SCLC cases, while successively trading specificity for sensitivity.

1. Introduction

Pulmonary small cell carcinoma (SCLC) can be usually diagnosed based on morphological evaluation of routine histological or cytological preparations. However, evaluation by immunohistochemistry (IHC) may be necessary to confirm epithelial and neuroendocrine differentiation in problematic cases, which include small biopsy specimens with extensive crush artifacts or scant cytological material [1].

Insulinoma-associated 1 (INSM1) is a transcription factor that plays a pivotal role in the development and differentiation of pancreatic and gastrointestinal neuroendocrine cells, adrenal medulla, and neuronal progenitor cells [2]. In normal tissues, INSM1 is expressed by neuroendocrine cells, such as those in the gastrointestinal, pancreatic, bronchopulmonary, and adrenal medullary tissues, but not in the normal brain parenchyma, normal parathyroid, or other normal tissues [2-4].

Recent studies demonstrated expression of INSM1 in a variety of low-grade and high-grade nonparathyroid neuroendocrine tumors, central nervous system neoplasms, and extraskeletal myxoid chondrosarcoma [2-8]. In the context of thoracic and pulmonary pathology, INSM1 has been reported as a highly sensitive and specific neuroendocrine marker that outperforms the conventional triad of synaptophysin (SYP), chromogranin A, and CD56, being positive in all well-differentiated neoplasms (typical carcinoids, atypical carcinoids, and paragangliomas) and in ~90%-100% of poorly differentiated tumors (small cell and large cell neuroendocrine carcinomas) [5,6]. Consequently, INSM1 was suggested to serve as a sufficiently sensitive and specific first-line marker of neuroendocrine differentiation, although this suggestion was based on a relatively small number of investigated cases [6].

In our previous study, we examined the expression of p16, CD56, SYP, chromogranin A, and thyroid transcription factor-1 (TTF1) in a series of pulmonary and extrapulmonary small cell carcinomas, and we compared diagnostic performance of these markers in the diagnosis of SCLC. We found that composite panels of only 3 markers, CD56 + p16 + TTF1 and CD56 + p16 + SYP, were both able to detect correctly all the SCLC cases. Of these 2 composite markers, we argued that the former is likely more proficient than the latter, considering that we showed higher individual sensitivity of TTF1 versus SYP but also because of the fact that CD56 and TTF1 were less correlated and likely more independent markers of SCLC than CD56 and SYP [9].

The aim of this study was to compare diagnostic performance of INSM1 and our previously reported composite marker CD56 + p16 + TTF1 in the diagnosis of SCLC in small biopsy specimens and cytoblocks.

2. Materials and methods

The study group consisted of 112 SCLC cases (104 primary and 8 metastatic). Of these cases, 69 were men and 43 were women, and their median age was 67 years (range 41-88 years). Paired endoscopic/core needle biopsy and cytoblock specimens were available for 9 patients, making 121 specimens available for immunohistochemical analysis. Some of these cases have been included in our previous study [9]. The study was conducted following the rules set by the Faculty Hospital in Pilsen Ethics Committee.

All cases were examined by IHC for p16, CD56, TTF1, and INSM1 expression. The immunohistochemical study was performed using a Ventana Benchmark XT automated stainer (Ventana Medical System, Inc, Tucson, AZ) using the following primary antibodies: p16 (R 19-D, monoclonal, DB Biotech, Košice, Slovakia, 1:300), TTF1 (SPT24, monoclonal, Novocastra, Leica Biosystems, Newcastle Upon Tyne, United Kingdom, 1:300), CD56 (1B6, monoclonal, Novocastra, Leica Biosystems, Newcastle Upon Tyne, United Kingdom, 1:400), and INSM1 (A-8, monoclonal, Santa Cruz Biotechnology, Dallas, TX, 1:1000), using either diaminobenzidine (p16, TTF1, CD56), or alkaline phosphatase (INSM1) as a chromogen. Appropriate positive and negative controls were used.

Proportions of positive cells for each marker were determined, and the findings were scored as positive for TTF1, CD56, or p16 if ≥5% corresponding positive cells were found [9]. For INSM1, findings were considered positive if any positive cells were found [6].

Sensitivities of CD56, TTF1, p16, and our composite marker CD56 + TTF1 + p16 (combined in parallel) to diagnose SCLC in our set of 100 endoscopic biopsies were compared with sensitivity of INSM1 marker using McNemar test. The performance of these markers to diagnose SCLC was further evaluated on 13 cytoblock specimens and 8 specimens from metastatic SCLC cases. Ninety-five percent confidence intervals (CIs) for proportions were calculated using Wilson's method with continuity correction [10]. Concordance between proportions of positive cells between 9 paired biopsy and cytoblock specimens was evaluated by Lin and Torbeck's [11] concordance correlation. Agreement between classifications of paired biopsy and cytoblock specimens as positive or negative was evaluated for each marker using Cohen κ coefficient. Statistical evaluation was performed using MedCalc Statistical Software version 17.9.7 (MedCalc Software, Ostend, Belgium). The differences were considered statistically significant for $P < .05$.

3. Results

Representative SCLC endoscopic biopsy and cytoblock specimens stained for each marker are presented in Figs. 1 to 3.

The proportion of INSM1-positive endoscopic biopsy specimens among primary SCLC cases was found to be 81% (95% CI = 71.7%-87.9%). Interestingly, CD56, TTF1, and p16 each individually classified more specimens correctly than INSM1, with the following proportions of positive specimens: CD56: 84% (95% CI = 75.0%-90.3 ;%), TTF1: 89% (95% CI = 80.8%-94.1%), and p16: 95% (95% CI = 88.2%-98.1%). However, the difference was statistically significant only for

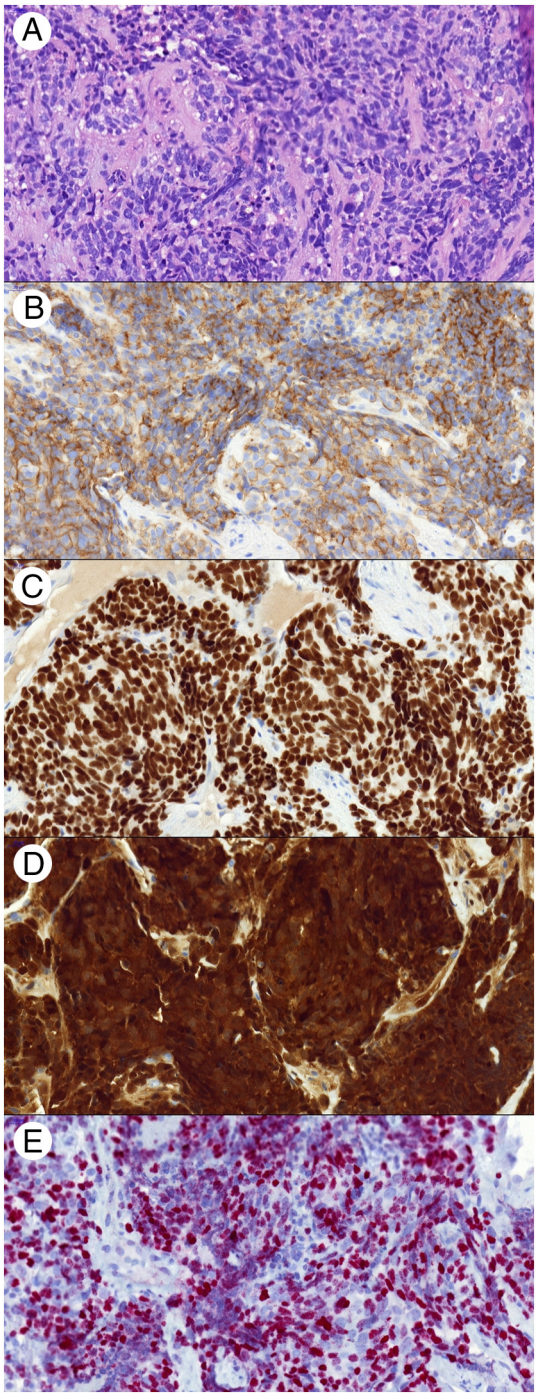

Fig. 1 Positivity for all 4 markers is present in this endoscopic biopsy specimen showing almost no crush artifact. A, Hematoxylin-eosin, (B) CD56, (C) TTF1, (D) P16, and (E) INSM1. Original magnification (A-E) ×400.

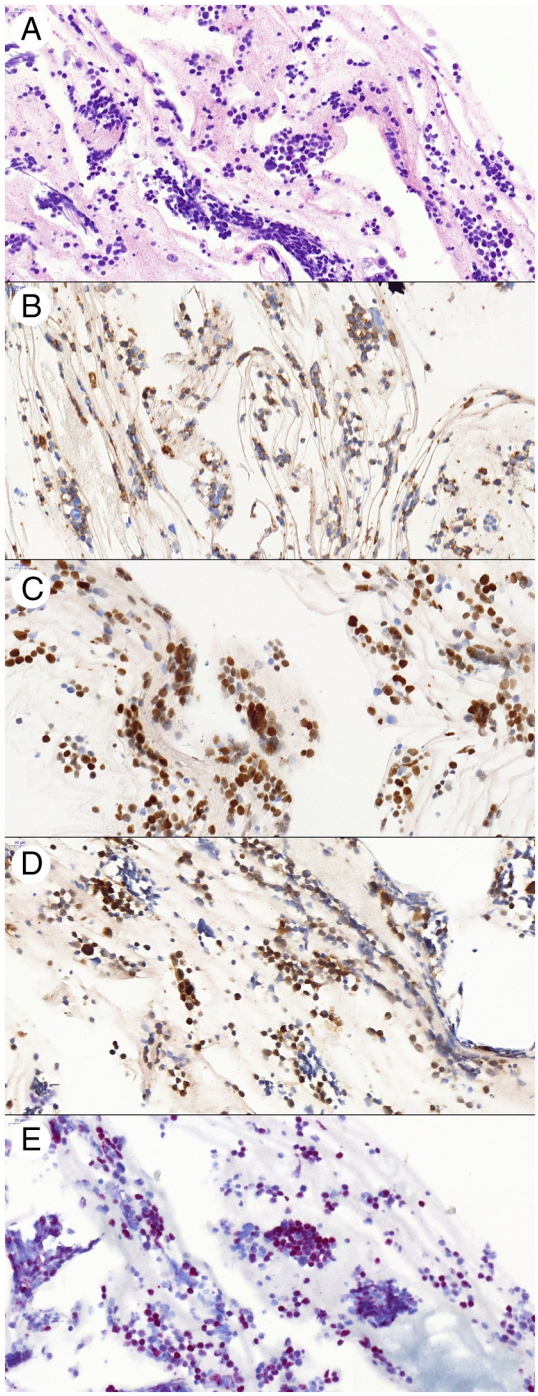

Fig. 2 Positive expression of all 4 markers in a cytoblock specimen. In cytoblocks, sometimes, it is difficult to differentiate between the tumor cells, the nuclei of respiratory cells, and lymphocytes. A, Hematoxylin-eosin, (B) CD56, (C) TTF1, (D) P16, and (E) INSM1. Original magnification (A-E) ×400.

p16 (Δ = 14%; 95% CI[Δ] = 6.2%-21.9%; P = .0013). Cross-classifications of endoscopic biopsy specimens used in the McNemar test are presented in Tables 1-4.

Combined marker CD56 + TTF1 + p16 (used in parallel) correctly classified 100% of our SCLC cases, and its sensitivity was significantly higher than the sensitivity of INSM1 (Δ = 19%; 95% CI[Δ] = 11.3%-26.7%; P < .0001).

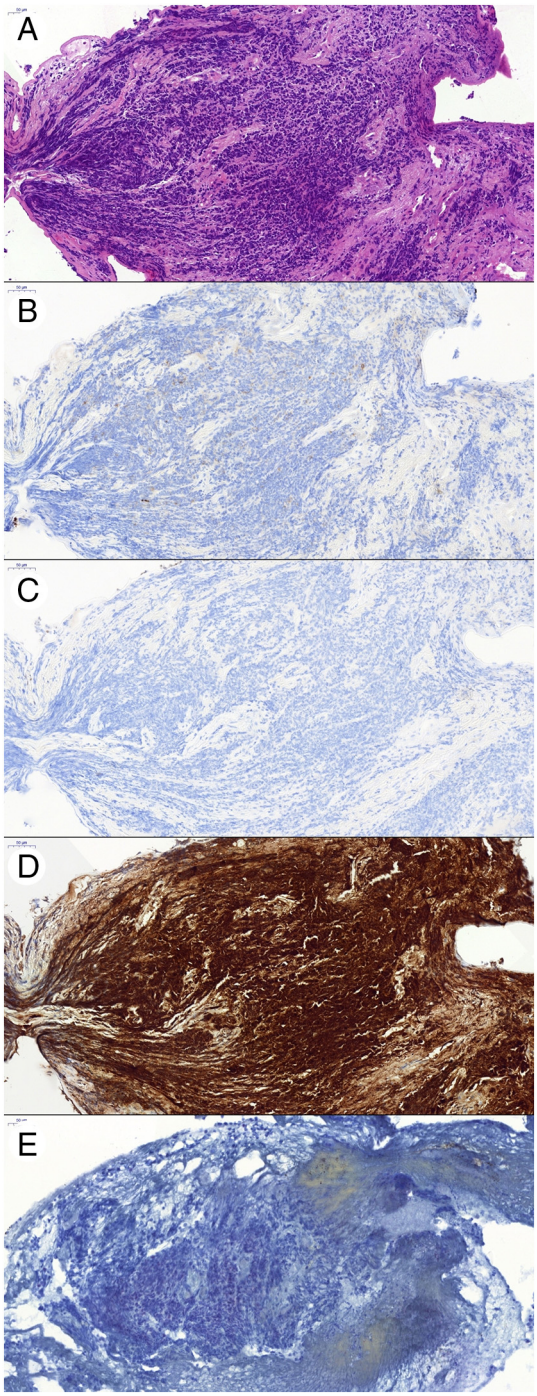

Fig. 3 A, In this study, P16 alone displayed significantly higher sensitivity to detect SCLC than INSM1, as demonstrated in this endoscopic biopsy showing severe crush artifact (hematoxylin-eosin). This case showed P16 expression only (D) and was negative for CD56 (B), TTF1 (C), and INSM1 (E). Original magnification (A-E) ×200.

Table 1 Cross-classification of 100 SCLC endoscopic biopsy specimens for INSM1 and p16 immunoreactivity

	INSM1+	INSM1−	Σ
p16+	79	16	95
p16−	2	3	5
Σ	81	19	100

Table 2 Cross-classifications of 100 SCLC endoscopic biopsy specimens for INSM1 and TTF-1 immunoreactivity

	INSM1+	INSM1−	Σ
TTF-1+	78	11	89
TTF-1−	3	8	11
Σ	81	19	100

Among all evaluated markers, INSM1 showed the lowest concordance of percent positive cells between paired endoscopic biopsy and cytoblock specimens (Table 5). The result suggests imprecision rather than a systematic difference between the paired biopsy and cytoblock specimens with respect to proportions of INSM1-positive cells. To establish whether the differences in concordances of percent positive cells between paired endoscopic biopsy and cytoblock specimens result in different positive/negative classification of these paired specimens for any given marker, we determined the degree of agreement between the classifications by Kohen κ. CD56 and p16 displayed perfect classification agreement between the paired specimens (for CD56: 9 positive/9 total; for p16: κ = 1), whereas TTF1 showed moderate agreement (κ = 0.4) and INSM1 showed only a slight agreement (κ = 0.182). Consequently, the result implies that lower concordance between percent positive cells for p16 relative to TTF1 (Table 5) did not project into lower agreement between p16-positive versus p16-negative classification of the paired

Table 3 Cross-classifications of 100 SCLC endoscopic biopsy specimens for CD56 and INSM1 immunoreactivity

	INSM1+	INSM1−	Σ
CD56+	70	14	84
CD56−	11	5	16
Σ	81	19	100

Table 4 Cross-classifications of immunoreactivity of 100 SCLC endoscopic biopsy specimens for INSM1 and for composite marker CD56 + TTF-1 + p16 (parallel)

	INSM1+	INSM1−	Σ
(CD56 + TTF-1 + p16)+	81	19	100
(CD56 + TTF-1 + p16)−	0	0	0
Σ	81	19	100

Table 5 Concordance in percent positive cells between paired endoscopic biopsy and cytoblock specimens for SCLC cases

	Concordance correlation coefficient (95% CI)	Pearson ρ	Bias correction factor
CD56	0.703 (0.142-0.922)	0.711	0.989
TTF-1	0.615 (0.011-0.890)	0.646	0.953
P16	0.388 (−0.286 to 0.805)	0.409	0.947
INSM1	0.209 (−0.443 to 0.716)	0.225	0.927

specimens. On the other hand, our finding of the lower agreement between paired specimens for INSM1 staining implies that a reliance solely on this marker for diagnosis of SCLC would more frequently fail to establish correlation between biopsy and cytology when both specimens are available for evaluation (we found 3 discordant results in our set of 9 paired specimens).

Of 8 metastatic SCLC cases, 7 were correctly classified by any evaluated marker, and 1 case, which represented SCLC metastasis to the lymph node, showed negativity for each of these markers. Positivity for individual markers in cytoblock specimens was found as follows: 13/13 for CD56, 11/13 for p16, 10/13 for TTF1, and only 7/13 for INSM1. The difference between sensitivity of CD56 and INSM1 for diagnosis of SCLC in cytoblock specimens is significant (Δ = 46.2%, 95% CI[Δ] = 19.1%-73.3%; $P < .031$).

4. Discussion

Histopathological diagnosis of SCLC is usually straightforward, but challenging cases need diagnostic support by immunohistochemistry. Recently, a strategy for IHC in the diagnosis of SCLC was described by an international panel of lung pathologists [1]. The first step included a "pro-SCLC" panel consisting of 1 or more of the following antibodies: CD56, low–molecular weight cytokeratin (eg, CK7, CK8, CK8/18), TTF1, MIB1, and perhaps p16 [1]. Synaptophysin, TTF1, and CD56 are reportedly the best and the most commonly used positive IHC markers for SCLC, being positive in up to 96% and 100% of cases, respectively [1,12-24]. However, with the exception of SYP, these markers are not considered specific for SCLC or neuroendocrine differentiation, and the well-established neuroendocrine marker SYP was found to be positive in some cases of morphologically conventional NSCLC [9,25]. On the other hand, chromogranin A, which is regarded as the most specific marker of neuroendocrine differentiation, displays low sensitivity because its expression may vary with degree of tumor differentiation, and focal, week, or even absent staining may be noted in poorly differentiated neuroendocrine carcinomas [12,13,18,20]. Considering these limitations, the development of new diagnostic markers for SCLC is still warranted [26]. One of the novel prospective markers of SCLC is INSM1, which was recently shown to display higher sensitivity for diagnosis of SCLC and large cell neuroendocrine carcinoma (LCNEC) than traditional neuroendocrine markers CD56, chromogranin A, and SYP, individually or combined [6]. However, in our group of 100 SCLC cases, CD56, TTF1, and p16 each correctly classified more biopsy specimens than INSM1. In fact, both p16 alone and our previously reported composite marker CD56 + TTF1 + p16 displayed significantly higher sensitivity to detect SCLC than INSM1 [9]. This result further supports the use of this composite marker for diagnosis of SCLC. On the other hand, INSM1 appears to show higher specificity, and it reportedly shows positivity in a considerably lower proportion of NSCLC cases [6,26,27] than p16 or the composite biomarker CD56 + TTF1 + p16 [9]. The results presented in this study suggest that INSM1 is a more sensitive marker for diagnosis of SCLC than other neuroendocrine markers chromogranin A and SYP reported in our previous study [9].

Among 100 primary SCLC cases, for which biopsy specimens were available, 19 cases were not properly classified by INSM1. Of them, 16 cases could be properly classified by p16, 14 cases by CD56, and 11 cases by TTF1 (using ≥5% positive cells as threshold). We have previously reported CD56 as the best performing single marker for differential diagnosis of SCLC and NSCLC, which at 15% threshold of positivity displayed sensitivity of 80.2% and specificity of 98.0% [9]. In this study, combination of INSM1 (0% threshold) with CD56 (15% threshold) correctly classified 94/100 specimens as SCLC, which is comparable with p16, the most sensitive single marker in this study (95/100; Δ = −5.5% to 7.5%, P = 1). Thus, parallel combination of INSM1 + CD56 appears to be a marker with high sensitivity comparable with p16 but presumably also with much higher specificity, and this combination warrants further evaluation. Nevertheless, sensitivity of the composite marker CD56 + TTF1 + p16 is still significantly higher than that of CD56 + INSM1 (100% versus 94%; Δ_{95} = 1.4%-10.7%; P = .0313).

In conclusion, although INSM1 is reportedly the most specific marker of SCLC, its sensitivity is not superior to p16 or composite marker CD56 + TTF1 + p16. Based on this study, the following approach in the diagnosis of SCLC may be proposed. First, INSM1 detection by IHC is performed. If the result is negative, CD56 is added, followed successively by p16 and TTF1 if all previously applied markers showed negative staining. This approach should detect most, if not all, SCLC cases, while successively trading specificity for sensitivity. Negative result for all these markers will rule out diagnosis of SCLC with high confidence. On the other hand, positive result of the proposed IHC algorithm will be supportive for SCLC but not diagnostic by itself because none of these markers provides high specificity for the detection of neuroendocrine differentiation or diagnosis of SCLC. Although INSM1 is expected to be the most specific component of the proposed

algorithm for the diagnosis of SCLC, this specificity applies to the diagnosis of SCLC versus non-neuroendocrine NSCLC, and this marker would not distinguish SCLC from LCNEC, or typical and atypical carcinoids [6].

Consequently, to rule out LCNEC and typical or atypical carcinoids, the results of IHC need to be interpreted in the context of morphology, frequency of mitoses, or proliferation index (Ki-67) when counting of mitoses is not possible because of the presence of severe crush artifacts. To rule out other possible diagnoses, such as poorly differentiated (basaloid) squamous cell carcinoma or lymphoma, other staining, such as p40/p63 or CD45, needs to be used [1,21,28-32].

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