Preprint (submitted version) of Zombori T, Turkevi-Nagy S, Sejben A, Juhász-Nagy G, Cserni G, Furák J, Tiszlavicz L, Krenács L, Kővári B. The panel of syntaxin 1 and insulinoma-associated protein 1 outperforms classic neuroendocrine markers in pulmonary neuroendocrine neoplasms. APMIS, First published: 08 January 2021 https://doi.org/10.1111/apm.13113 for the SZTE non-commercial institutional repository

Syntaxin 1, a superior marker of pulmonary neuroendocrine neoplasms – the panel of Syntaxin 1 and Insulinoma-Associated Protein 1 outperforms classic neuroendocrine markers

Authors: Tamás Zombori[1], Sándor Turkevi-Nagy[1], Anita Sejben[1], Gréta Juhász-Nagy[1], Gábor Cserni[1,2], József Furák[3], László Tiszlavicz[1], László Krenács[4]*, Bence Kővári[1]*

1 Department of Pathology, University of Szeged, Szeged, Hungary

2 Bács-Kiskun County Teaching Hospital, Kecskemét, Hungary

3 Department of Surgery, University of Szeged, Szeged, Hungary

4 Laboratory of Tumor Pathology and Molecular Diagnostics, Szeged, Hungary

* László Krenács and Bence Kővári equally contributed to this work and its supervision

Phone numbers and email addresses of the authors:

Tamás Zombori: +36-30-8447190; zomtam@gmail.com

Sándor Turkevi-Nagy: +36-62-545-878; tenagysanyi@gmail.com

Anita Sejben: +36-62-545-878, sejben.anita@gmail.com

Gréta Juhász-Nagy: +36-62-545-878, aterg94@gmail.com

Gábor Cserni: +36-62-545-878, csernig@hotmail.com

József Furák: +36-62-545-445, jfurak@gmail.com

László Tiszlavicz: +36-62-545-878, tiszlats@yahoo.com

László Krenács: +36-62-545-878, krenacsl@vipmail.hu

Bence Kővári: +36-62-545-878, kovari.bence.p@gmail.com

Running title: Syntaxin an emerging neuroendocrine marker

Summary:

Tamás Zombori[1], Sándor Turkevi-Nagy[1], Anita Sejben[1], Gréta Juhász-Nagy[1], Gábor Cserni[1,2], József Furák[3], László Tiszlavicz[1], László Krenács[4]*, Bence Kővári[1]. **Syntaxin 1, a superior marker of pulmonary neuroendocrine neoplasms – the panel of Syntaxin 1 and Insulinoma-Associated Protein 1 outperforms classic neuroendocrine markers**

Syntaxin-1 (STX1) is a recently described highly sensitive and specific neuroendocrine marker. In this study, we aimed to evaluate the applicability of STX1 as an immunohistochemical marker in pulmonary neuroendocrine neoplasms (NENs). We also compared STX1 with established neuroendocrine markers, including insulinoma-associated protein 1 (INSM1).

Typical carcinoids (TC, n=33), atypical carcinoids (AC, n=7), small cell lung carcinomas (SCLC, n=28), and large cell neuroendocrine lung carcinomas (LCLNC, n=17) were immunostained using tissue microarray for STX1, Chromogranin-A (CHGA), synaptophysin (SYP), CD56, and INSM1.

All NENs, but one SCLC (84/85 cases), showed STX1 positivity. Carcinoids and LCNCLs, typically presented a combined strong membranous and weak cytoplasmic staining pattern, whereas predominantly cytoplasmic expression was observed in SCLCs. The sensitivity of STX1 was 96.4% in SCLCs and 100% in TCs, ACs, and LCLNCs. The overall sensitivity of STX1 in pulmonary NENs was 98.8%, while the sensitivity of the other markers was as follows: CHGA (89.3%), SYP (89.3%), CD56 (95.2%), and INSM1 (97.6%).

STX1 was found to be an excellent neuroendocrine marker of pulmonary NENs, surpassing classic markers in terms of sensitivity and specificity. We propose a panel of STX1 and INSM1 for the routine immunohistochemical workup of pulmonary NENs.

Keywords: syntaxin-1; insulinoma-associated protein 1; pulmonary neuroendocrine neoplasms; small cell carcinoma; immunohistochemistry.

Correspondence: Tamás Zombori, MD, PhD

Phone: +36308447190,
E-mail: zomtam@gmail.com

ORCID ID: https://orcid.org/0000-0002-0654-563X

Introduction

The lung and the gastrointestinal tract are the most frequent sites of neuroendocrine (NE) neoplasms (NEN) (1). From the two regions, the lung is the single most commonly affected organ, with a high proportion of poorly differentiated neoplasms. According to the most recent WHO classification of lung tumors (2), the pulmonary NENs are classified as small cell carcinomas (SCLC), large cell neuroendocrine carcinomas (LCNEC), typical carcinoids (TC), and atypical carcinoids (AC). Although these tumors have characteristic morphological features (*Fig. 1*), given the specific clinical management of NENs, the verification of NE nature is recommended by immunohistochemical (IHC) detection of characteristic antigens (2, 3). Chromogranin-A (CHGA), synaptophysin (SYP), and CD56 are the most widely used NE IHC markers, and insulinoma-associated protein 1 (INSM1) is also increasingly applied in the diagnostic pathology work-up. Nevertheless, pathologists should be aware of their limitations (2, 4).

CHGA is a major component of NE secretory vesicles along with chromogranin-B and chromogranin-C. CHGA is prevalently expressed in normal NE cells and NENs, however the intensity of expression depends on the density of neurosecretory granules and the balance between the different chromogranins (5). In poorly differentiated neoplasms, such as SCLC and LCLC, the quantity of these granules may be very low, resulting in weak and focal paranuclear dot-like staining (1). The expression rate of CHGA is 23% to 58% in SCLCs and 42% to 69% in LCNECs (5-14).

SYP is a calcium-binding glycoprotein in the synaptic microvesicles of normal and neoplastic NE cells, and it is expressed in 41% to 75% of SCLCs and 58% to 85% of LCNECs (15). Although SYP is present in both poorly and well-differentiated NENs, unfortunately, non-NE tumors may also be labelled (15, 16).

CD56 or neural cell adhesion molecule (N-CAM) was demonstrated on the membrane of a wide range of human cells, including natural killer cells and T lymphocytes, epithelium of the thyroid, kidney, and adrenal cortex, as well as skeletal muscle, and NE tissues (17). Despite its high sensitivity for NE differentiation, CD56 can show positivity in a plethora of diverse neoplasms, substantially limiting its specificity (17-23).

INSM1, an increasingly recognized NE marker, represents a zinc-finger transcription factor, first identified in pancreatic insulinoma (24). INSM1 was demonstrated as a

controller of the CHGA and SYP expression, a regulator of the cell cycle (25, 26), having a vital role in the physiologic development of NE tissues throughout the body (27). Rooper and coworkers described INSM1 as a reliable marker of NE differentiation among lung NENs with an excellent sensitivity of 96.4%, however some non-NE pulmonary neoplasms expressed INSM1 focally (4).

Syntaxin-1 (STX1) is a member of a complex synaptic protein superfamily and plays a key role in calcium-dependent exocytosis of neurotransmitters in the nervous and NE system. STX1 possesses a C-terminal transmembrane domain, namely SNARE (Soluble NSF (N-ethylmaleimide-sensitive fusion protein)-Attachment protein Receptor), which takes part in the SNARE-dependent membrane fusion and docking of neurosecretory vesicles (28). We recently found that STX1 represents a powerful NE marker, possessing an outstanding sensitivity (97%) and specificity (100%) in a very wide variety of NENs (29). In this study, we aimed to assess systematically STX1 expression in pulmonary NENs regarding extent, intensity, and staining pattern.

Materials and methods

Lung specimens surgically resected between 2003-2019 were collected from the files of the Department of Pathology, University of Szeged, Hungary. SCLCs (n=28), LCNLCs (n=17), TCs (n=33), and ACs (n=7) were included in our retrospective and consecutive series. One of the TC cases emerged in the background of diffuse idiopathic pulmonary NE cell hyperplasia (DIPNECH). The morphology of the selected cases was re-evaluated according to the 2015 edition of the WHO classification of lung tumors (2). Accordingly, at least one of the three markers (CHGA, SYP, CD56) had to be positive in the context of a proper NE histomorphology for inclusion in the present series. Representative paraffin blocks were collected for tissue microarray (TMA) and two 2.2 mm diameter core samples were taken from each case. Additional positive and negative controls were included in each TMA block.

The IHC reactions were uniformly performed on formalin-fixed and paraffin-embedded sections, following the protocols described in our earlier publication (29). For STX1, we used the mouse monoclonal antibody HPC-1 (sc-12736; Santa Cruz Biotechnology, Dallas, TX, USA; 1:200), that detects both STX1 A and B isoforms. INSM1 reactions were prepared using a mouse monoclonal antibody A8 (Santa Cruz, city, country; 1:100). Primary antibodies and the protocols used for the various IHC reactions are

detailed in Supplementary table 1. The TMA slides were evaluated by two observers after an initial training session; finally, discrepancies were discussed.

The extent of expression, the intensity, the cellular localization, and the sensitivity of STX1, CHGA, SYP, CD56 and INSM1 were evaluated. Membranous or cytoplasmic staining for STX1 and CD56, cytoplasmic staining for CHGA and SYP, finally, nuclear staining for INSM1 in more than ≥5% of tumor cells was considered positive. The positivity was regarded diffuse if more than 75% of the tumor cells were labelled. Concerning staining intensity, three categories were applied, namely weak (1+), moderate (2+), and strong (3+). If a case was negative or <5% positive for any of the classic NE markers (CHGA, SYP or CD56) in the TMA cores, the available original diagnostic whole block IHC reactions were also re-evaluated and discussed.

The sensitivity of STX1, CHGA, SYP, CD56, and INSM1 was calculated for TCs and ACs, as well as for SCLCs and LCNLCs. Additionally, for the direct comparison of the sensitivities of STX1 and other NE markers, we indirectly correlated our results to immunophenotyping data of CHGA, SYP, CD56, and INSM1 from recent publications evaluating pulmonary NENs (Supplementary table 2) (4, 30-36). The data gathered from the literature review were used to calculate the average sensitivities and the ranges of sensitivity of CHGA, SYP, CD56, and INSM1.

The study was approved by the Ethical Review Board of the University of Szeged (#4430/2018).

Results

Altogether 85 patients were included in our retrospective study. There was no gender predilection (female : male=1:1). Supplementary table 3 highlights the further relevant clinical and pathologic features of the evaluated cases.

All NENs except a single case of SCLC showed STX1 expression. Table 1 shows the results concerning the extent, and the intensity of STX1 expression. Diffuse positivity was detected in 80/85 cases (94%); whereas focal staining (range: 5-75%) was present in two SCLCs, one LCNLC, and one TC.

The median intensity of STX1 labeling was strong (3+) in ACs and SCLCs, whereas moderate (2+) in TCs and LCNLCs (Table 1).

Table 2 demonstrates the cellular localization of STX1 expression in tumor cells. Predominant membranous staining with weak cytoplasmic labeling was the most

frequent pattern among TCs, ACs, and LCNLCs (*Fig. 1*), while cytoplasmic expression of STX1 was seen mostly in SCLCs and a minority of LCNLCs. Nuclear staining of STX1 was not observed. In the DIPNECH associated TC case, both the preinvasive and the invasive components showed strong diffuse positivity with a combined membranous and cytoplasmic localization.

As concerns the other NE markers, Table 1 also shows the extent and intensity of positivity for CHGA, SYP, CD56, and INSM1. Diffuse INSM1 positivity was registered in 66/85 cases (77.6%); while focal staining (range: 10-75%) was observed in 8 SCLCs, 6 LCNLCs, and 5 TCs. In the rare cases with only focal STX1 and INSM1 staining, the expression always exceeded the 5% cut-off. Scattered positivity below the 5% cut-off only occurred using CHGA, SYP, and CD56, rendering those cases negative (Table 3). The STX1 negative SCLC was originally considered NE based on morphology and CD56 expression and was also negative for CHGA and SYP. This case demonstrated limited positivity with INSM1 (Table 3). The median intensity of INSM1 positivity was strong in both carcinoids and high-grade NENs; while CHGA and SYP tend to label carcinoids strongly, but NE carcinomas only moderately. In keeping with literature data, the staining pattern was exclusively nuclear for INSM1, cytoplasmic for SYP and CHGA, and combined membranous and cytoplasmic for CD56 in all pulmonary NEN subtypes.

The sensitivity of STX1 was 97.8% (95%CI:0.88-0.99) among high-grade pulmonary NENs and 100% (95%CI:0.91-1) among pulmonary carcinoids. The overall sensitivity of STX1 for NE differentiation proved to be 98.8% (95%CI:0.93-0.99), while the sensitivity of the other evaluated NE markers was as follows: CHGA (89.3%), SYP (89.3%), CD56 (95.2%), and INSM1 (97.6%). Table 1 also demonstrates the detailed sensitivity data of these NE markers regarding all individual pulmonary NEN subtypes.

Discussion

The evolution of therapeutic modalities requires the permanent refinement of diagnostic methods, including ancillary studies in histopathology.

As pathologists are keen on providing accurate preoperative diagnosis based on tiny biopsy samples, the recent approach demands the application of IHC in cases of tumors without obvious morphological features of glandular, squamous or NE differentiation. Furthermore, considering that the clinical management of pulmonary NENs differ from

other types of neoplasms, and the precise identification of the NE nature is necessary for the proper treatment, it is better to perform IHC in all cases with the possibility of NE differentiation (1, 37, 38).

The sensitivity and the specificity of CHGA, SYP, and CD56 are not perfect. CHGA and SYP are specific, but can lack sensitivity in certain settings, while CD56 is sensitive, but cannot be considered a specific marker of NE nature. These discrepancies cause potential diagnostic pitfalls when it comes to the diagnosis of challenging pulmonary NENs (1, 39-41), therefore research for new, possibly more reliable IHC markers is required.

A potential novel NE marker is STX1, a member of the syntaxin family, that takes part in the calcium-modulated, SNARE-dependent membrane fusion of neurons and NE cells (28, 42). In our series, 84/85 pulmonary NENs were positive for STX1, furthermore the positivity was diffuse in more than 90% of cases. The degree of differentiation may influence the extent, the distribution and the localization of protein expression in tumors. This phenomenon is not unusual among NE carcinomas, as the density of mature secretory granules is frequently decreased, resulting in only focal CHGA expression (1, 43). Although, there were some differences between the STX1 expression patterns of low/intermediate-grade and high-grade pulmonary NENs, diffuse positivity was still present in approximately 90% of SCLCs and LCNLCs. Nevertheless, the only STX1 negative case was an SCLC, and the extent of expression was also somewhat lower in NE carcinomas. A strong membranous accentuation of STX1 expression was characteristic of TCs, ACs, most LCNLCs, and the single case of DIPNECH, while primarily cytoplasmic staining pattern was observed in SCLCs and in a minority of LCNLCs. After the comparison of all tested NE markers, STX1 showed the smallest difference between carcinoids and NE carcinomas regarding the extent of expression, while INSM1 showed the smallest difference concerning the intensity of labeling.

Our findings suggest that the sensitivity of STX1 among pulmonary NENs is perfect (100%) in TCs, ACs, and LCNLC, while 96.4% in cases of SCLC. A comprehensive comparison was conducted between the sensitivity data of the classic WHO recommended (CHGA, SYP, and CD56) and novel (STX1 and INSM1) NE markers; that incorporated both the direct comparison of our IHC results (Table 1) and an indirect comparison based on a literature review (Table 4 and Supplementary table 2) (4, 30-36). The overall sensitivity of STX1 (98.8%) was superior to the sensitivity of CHGA

(direct: 89.3%; indirect: 75.1%), SYP (direct: 89.3%; indirect: 86.6%) CD56 (direct: 95.2%; indirect: 92.7%), and INSM1 (direct: 97.6%; indirect: 85.6%) irrespective of the modality of comparison.

Although non-NE tumors were not evaluated in the present study, in this regard we refer to our previous research (29), which encompassed a wide spectrum of non-NE neoplasms including pulmonary squamous cell carcinomas and pulmonary adenocarcinomas, as well as colorectal, hepatocellular, pancreatobiliary, head and neck, thyroid, adrenocortical, skin basal cell, uterine, cervical, ovarian, and prostatic carcinomas. The non-NE carcinomas and the non-NE areas of mixed NE-non-NE carcinomas were generally negative for STX1. STX1 expression can also be present in medulloblastomas, neuroblastic tumors, paragangliomas, pheochromocytomas, and in the NENs of other organs (29), therefore one should correlate the IHC findings to histomorphology and clinical data for the correct interpretation of protein expression. Fortunately, given the considerably different clinicopathological presentation of medulloblastomas and neuroblastic tumors, positivity in these tumors should rarely result in diagnostic errors. Overall, the specificity of STX1 has been found excellent (99.41% 95%CI:0.96-0.99), even somewhat better than that of INSM1 (96.3% 95%CI:0.92-0.98) (4, 29).

Altogether STX1 and INSM1 mutually have promising sensitivity and specificity and should be recommended for routine diagnostic application. Although, Rooper et al. demonstrated focal INSM1 expression in pulmonary adenocarcinomas and squamous cell carcinomas (4), such a phenomenon was not found with STX1 in the limited series of Kővári et al. (29). In our opinion, the nuclear expression of INSM1 and the frequently crisp membranous quality of STX1 labeling can be more consistently interpreted, than the cytoplasmic staining for SYP and CHGA, the latter of which could be harder to distinguish from unspecific background staining in case of weak and focal expression. Due to the relative shortage of tissue in small biopsy specimens and financial reasons, the panel of NE markers could not be extended. Based on the presented data a panel of STX1 and INSM1 could outperform the currently recommended CHGA, SYP, and CD56 panel (Table 3). Owing to the different intracellular localization of staining, STX1 and INSM1 can also be used in combined IHC reactions to spare biopsy material which might often be of limited amount. Our results also raise the possibility that STX1 IHC staining alone may be sufficient for the diagnosis of NE differentiation.

Limitations of this study could be the relatively low case number and the use of the TMA method. Even though the TMA method may lead to misinterpretation of focal staining patterns due to tumor heterogeneity, Kővári and co-workers have demonstrated that STX1 expression is predominantly diffuse (29). Therefore, we considered the application of the TMA technique acceptable. The retrospective nature of our study is a potential source of selection bias, moreover the fact that the cases were originally diagnosed using the classic NE markers may have led to the overrepresentation of CHGA, SYP, and CD56 positive cases, and the overinterpretation of the sensitivities of these markers.

In conclusion, our retrospective, consecutive series aimed to evaluate the expression of STX1, a recently introduced NE marker among pulmonary NENs. STX1 was found to be a robust, easily interpretable and reliable marker with excellent sensitivity and specificity. Therefore, we encourage colleagues to apply STX1 as an IHC marker of NE differentiation in the routine diagnostics of pulmonary neoplasms. We feel, that after further investigation, STX1 and INSM1 may eventually replace the antibodies of the currently accepted panel of CHGA, SYP, and CD56.

***Acknowledgement*:** The authors thank photographer Mihály Dezső for his assistance with the figures. This work was supported by the University of Szeged, Faculty of Medicine Research Fund – Hetényi Géza grant 5S582.

References

1. Uccella S, La Rosa S, Volante M, Papotti M. Immunohistochemical biomarkers of gastrointestinal, pancreatic, pulmonary, and thymic neuroendocrine neoplasms. Endocr Pathol 2018;29:150-168.

2. Travis WD, Brambilla E, Burke AP, Marx A, Nicholson AG. WHO Classification of tumours of the lung, pleura, thymus and heart. Fourth edition. Lyon, International Agency for Research on Cancer, 2015.

3. Gosain R, Mukherjee S, Yendamuri SS, Iyer R. Management of typical and atypical pulmonary carcinoids based on different established guidelines. Cancers (Basel) 2018;10:510.

4. Rooper LM, Sharma R, Li QK, Illei PB, Westra WH. INSM1 demonstrates superior performance to the individual and combined use of synaptophysin,

chromogranin and CD56 for diagnosing neuroendocrine tumors of the thoracic cavity. Am J Surg Pathol 2017;41:1561-1569.
5. Tötsch M, Müller LC, Hittmair A, Ofner D, Gibbs AR, Schmid KW. Immunohistochemical demonstration of chromogranins A and B in neuroendocrine tumors of the lung. Hum Pathol 1992;23:312-6.
6. Barbareschi M, Girlando S, Mauri FA, Arrigoni G, Laurino L, Dalla Palma P et al. Tumour suppressor gene products, proliferation, and differentiation markers in lung neuroendocrine neoplasms. J Pathol 1992;166:343-50.
7. Hamanaka W, Motoi N, Ishikawa S, Ushijima M, Inamura K, Hatano S, et al. A subset of small cell lung cancer with low neuroendocrine expression and good prognosis: a comparison study of surgical and inoperable cases with biopsy. Hum Pathol 2014;45:1045-1056.
8. Jiang SX, Kameya T, Shoji M, Dobashi Y, Shinada J, Yoshimura H. Large cell neuroendocrine carcinoma of the lung: a histologic and immunohistochemical study of 22 cases. Am J Surg Pathol 1998;22:526-37.
9. Kaufmann O, Georgi T, Dietel M. Utility of 123C3 monoclonal antibody against CD56 (NCAM) for the diagnosis of small cell carcinomas on paraffin sections. Hum Pathol 1997;28:1373–1378.
10. Pelosi G, Rodriguez J, Viale G, Rosai J. Typical and atypical pulmonary carcinoid tumor overdiagnosed as small-cell carcinoma on biopsy specimens: a major pitfall in the management of lung cancer patients. Am J Surg Pathol 2005;29:179-87.
11. Shy SW, Lee WH, Chou MC, Lai YS, Tu YC. Small cell lung carcinoma: clinicopathological, immunohistochemical, and ultrastructural study. J Surg Oncol 1990;45:146-61.
12. Sturm N, Lantuéjoul S, Laverrière MH, Papotti M, Brichon PY, Brambilla C, et al. Thyroid transcription factor 1 and cytokeratins 1, 5, 10, 14 (34betaE12) expression in basaloid and large-cell neuroendocrine carcinomas of the lung. Hum Pathol 2001;32:918-25.
13. Tanaka Y, Ogawa H, Uchino K, Ohbayashi C, Maniwa Y, Nishio W, et al. Immunohistochemical studies of pulmonary large cell neuroendocrine carcinoma: a possible association between staining patterns with neuroendocrine markers and tumor response to chemotherapy. J Thorac Cardiovasc Surg 2013;145:839-46.

14. Wick MR, Berg LC, Hertz MI. Large cell carcinoma of the lung with neuroendocrine differentiation. A comparison with large cell “undifferentiated” pulmonary tumors. Am J Clin Pathol 1992;97:796–805.
15. Gould VE, Lee I, Wiedenmann B, Moll R, Chejfec G, Franke WW. Synaptophysin: a novel marker for neurons, certain neuroendocrine cells, and their neoplasms. Hum Pathol 1986;17:979-83.
16. Komminoth P, Roth J, Schröder S, Saremaslani P, Heitz PU. Overlapping expression of immunohistochemical markers and synaptophysin mRNA in pheochromocytomas and adrenocortical carcinomas. Implications for the differential diagnosis of adrenal gland tumors. Lab Invest 1995;72:424-31.
17. Shipley WR, Hammer RD, Lennington WJ, Macon WR. Paraffin immunohistochemical detection of CD56, a useful marker for neural cell adhesion molecule (NCAM), in normal and neoplastic fixed tissues. Applied Immunohistochemistry 1997;5:87-93.
18. Jin L, Hemperly JJ, Lloyd RV. Expression of neural cell adhesion molecule in normal and neoplastic human neuroendocrine tissues. Am J Pathol 1991;138:961-9.
19. Chu PG, Arber DA, Weiss LM. Expression of T/NK-cell and plasma cell antigens in nonhematopoietic epithelioid neoplasms. An immunohistochemical study of 447 cases. Am J Clin Pathol 2003;120:64-70.
20. Bösmüller HC, Wagner P, Pham DL, Fischer AK, Greif K, Beschorner C, et al. CD56 (Neural Cell Adhesion Molecule) expression in ovarian carcinomas: association with high-grade and advanced stage but not with neuroendocrine differentiation. Int J Gynecol Cancer 2017;27:239-245.
21. Vasei M, Moch H, Mousavi A, Kajbafzadeh AM, Sauter G. Immunohistochemical profiling of Wilms tumor: a tissue microarray study. Appl Immunohistochem Mol Morphol 2008;16:128-34.
22. Wachowiak R, Rawnaq T, Metzger R, Quaas A, Fiegel H, Kähler N, et al. Universal expression of cell adhesion molecule NCAM in neuroblastoma in contrast to L1: implications for different roles in tumor biology of neuroblastoma? Pediatr Surg Int 2008;24:1361-4.
23. Van Camp B, Durie BG, Spier C, De Waele M, Van Riet I, Vela E, et al. Plasma cells in multiple myeloma express a natural killer cell-associated antigen: CD56 (NKH-1; Leu-19). Blood 1990;76:377-82.

24. Goto Y, De Silva MG, Toscani A, Prabhakar BS, Notkins AL, Lan MS. A novel human insulinoma-associated cDNA, IA-1, encodes a protein with "zinc-finger" DNA-binding motifs. J Biol Chem 1992;267:15252-7.
25. Lan MS, Breslin MB. Structure, expression, and biological function of INSM1 transcription factor in neuroendocrine differentiation. FASEB J 2009;23:2024-2033.
26. Fujino K, Motooka Y, Hassan WA, Ali Abdalla MO, Sato Y, Kudoh S, et al. Insulinoma-associated protein 1 is a crucial regulator of neuroendocrine differentiation in lung cancer. Am J Pathol 2015;185:3164-77.
27. Gierl MS, Karoulias N, Wende H, Strehle M, Birchmeier C. The zinc-finger factor INSM1 (IA-1) is essential for the development of pancreatic beta cells and intestinal endocrine cells. Genes Dev 2006;20:2465-78.
28. Rizo J. Mechanism of neurotransmitter release coming into focus. Protein Sci 2018;27:1364-1391.
29. Kővári B, Turkevi-Nagy S, Báthori Á, Fekete Z, Krenács L. Syntaxin 1: A novel robust immunophenotypic marker of neuroendocrine tumors Int J Mol Sci 2020;21:1213.
30. Nicholson SA, Beasley MB, Brambilla E, Hasleton PS, Colby TV, Sheppard MN, et al. Small cell lung carcinoma (SCLC): a clinicopathologic study of 100 cases with surgical specimens. Am J Surg Pathol 2002;26:1184-97.
31. Yeh YC, Chou TY. Pulmonary neuroendocrine tumors: study of 90 cases focusing on clinicopathological characteristics, immunophenotype, preoperative biopsy, and frozen section diagnoses. J Surg Oncol 2014;109:280–286.
32. Doxtader EE, Mukhopadhyay S. Insulinoma-associated protein 1 is a sensitive and specific marker of neuroendocrine lung neoplasms in cytology specimens. Cancer Cytopathol 2018;126:243–252.
33. Kriegsmann K, Zgorzelski C, Kazdal D, Cremer M, Muley T, Winter H, et al. insulinoma-associated protein 1 (INSM1) in thoracic tumors is less sensitive but more specific compared with synaptophysin, chromogranin a, and CD56. Appl Immunohistochem Mol Morphol 2018;28:237-242.
34. Mukhopadhyay S, Dermawan JK, Lanigan CP, Farver CF. Insulinoma-associated protein 1 (INSM1) is a sensitive and highly specific marker of neuroendocrine differentiation in primary lung neoplasms: an immunohistochemical study of 345 cases, including 292 whole-tissue sections. Mod Pathol 2019;32:100–109.

35. Viswanathan K, Siddiqui MT, Borczuk AC. Insulinoma-associated protein 1 is a sensitive and specific marker for lung neuroendocrine tumors in cytologic and surgical specimens. J Am Soc Cytopathol 2019;8:299–308.
36. Staaf J, Tran L, Söderlund L, Nodin B, Jirström K, Vidarsdottir H, et al. Diagnostic value of insulinoma-associated protein 1 (INSM1) and comparison with established neuroendocrine markers in pulmonary cancers: a comprehensive study and review of the literature. Arch Pathol Lab Med 2020;144:1075–1085.
37. Kyriakopoulos G, Mavroeidi V, Chatzellis E, Kaltsas GA, Alexandraki KI. Histopathological, immunohistochemical, genetic and molecular markers of neuroendocrine neoplasms. Ann Transl Med 2018;6:252.
38. Lloyd RV. Practical markers used in the diagnosis of neuroendocrine tumors. Endocr Pathol 2003;14:293-301.
39. Kosuke F, Kazuhiro Y, Shinji K, Yamato M, Yonosuke S, Joeji W, et al. INSM1 is the best marker for the diagnosis of neuroendocrine tumors: comparison with chromogranin-A, SYP and CD56. Int J Clin Exp Pathol 2017;10:5393-5405.
40 Fahrenkamp AG, Wibbeke C, Winde G, Ofner D, Böcker W, Fischer-Colbrie R, et al. Immunohistochemical distribution of chromogranins A, B and secretogranin II in neuroendocrine tumours of the gastrointestinal tract. Virchows Arch 1995;426:361-7.
41. Al-Khafaji B, Noffsinger AE, Miller MA, DeVoe G, Stemmermann GN, Fenoglio-Preiser C. Immunohistologic analysis of gastrointestinal and pulmonary carcinoid tumors. Hum Pathol 1998;29:992–999.
42. Xie Z, Long J, Liu J, Chai Z, Kang X, Wang C. Molecular mechanisms for the coupling of endocytosis to exocytosis in neurons. Front Mol Neurosci 2017;10:47.
43. Klimstra DS. Pathology reporting of neuroendocrine tumors: essential elements for accurate diagnosis, classification, and staging. Semin Oncol 2013;40:23-36.
44. Rudin CM, Poirier JT, Byers LA, Dive C, Dowlati A, George J, et al. Molecular subtypes of small cell lung cancer: a synthesis of human and mouse model data. Nat Rev Cancer 2019;19:415.

Table 1 Extent and intensity of STX1, CHGA, SYP, CD56 and INSM1 expression and sensitivity according to NEN subtypes (TC: typical carcinoid, AC: atypical carcinoid, SCLC: small cell lung cancer, LCNLC: large cell neuroendocrine lung cancer, NEN: neuroendocrine neoplasm, 95%CI: 95% confidence interval)

		Extent		Intensity	Sensitivity (%)	95%CI
STX1	positive /all	average (%)	range (%)	median		
TC	33/33	92.66	10-100	2	100	0.89-1.00
AC	7/7	94.28	75-100	3	100	0.59-1.00
SCLC	27/28	81.14	5-100	3	96.4	0.82-0.99
LCNLC	17/17	79.81	10-100	2	100	0.80-1.00
NEN all	84/85	86.97	5-100	2	98.8	0.93-0.99
		Extent		Intensity	Sensitivity (%)	95%CI
CHGA		average (%)	range (%)	median		
TC	30/32	89.06	1-100	3	93.75	0.79-0.99
AC	7/7	95	100	3	100	0.59-1.00
SCLC	23/28	54.35	1-100	2	82.14	0.63-0.93
LCNLC	15/17	60.0	40-90	2	88.24	0.63-0.98
NEN all	75/84	72.25	1-100	2	89.3	0.80-0.94
		Extent		Intensity	Sensitivity (%)	95%CI
SYP		average (%)	range (%)	median		
TC	30/32	86.28	1-100	3	93.75	0.79-0.99
AC	7/7	87.14	1-100	3	100	0.59-1.00
SCLC	23/28	62	40-100	2	82.14	0.63-0.93
LCNLC	15/17	70.29	50-100	2	88.24	0.63-0.98
NEN all	75/84	75.02	1-100	2	89.3	0.80-0.94
		Extent		Intensity	Sensitivity (%)	95%CI
CD56		average (%)	range (%)	median		
TC	30/31	88.06	20-100	3	96.77	0.83-0.99
AC	7/7	87.86	70-100	3	100	0.59-1.00
SCLC	27/28	79.67	1-100	2	96.42	0.88-1.00
LCNLC	15/17	73.35	1-100	3	88.24	0.63-0.98
NEN all	79/83	82.2	1-100	3	95.18	0.88-0.98
		Extent		Intensity	Sensitivity (%)	95%CI
INSM1		average (%)	range (%)	median		
TC	29/31	80.16	30-100	3	93.55	0.78-0.99
AC	8/8	91.87	80-100	3	100	0.63-1.00
SCLC	27/27	80.74	20-100	2	100	0.87-1.00
LCNLC	17/17	77.05	10-100	3	100	0.80-1.00
NEN all	81/83	80.84	10-100	3	97.59	0.91-0.99

1

Table 2 Localization of STX1 expression according to different NEN subtypes (TC: typical carcinoid, AC: atypical carcinoid, SCLC: small cell lung cancer, LCNLC: large cell neuroendocrine lung cancer, NEN: neuroendocrine neoplasm, M: exclusive membranous, M/C: strong membranous and weaker cytoplasmic, C/M: weak membranous and stronger cytoplasmic, C: cytoplasmic)

	n (%)	**M**	**M/C**	**C/M**	**C**	**Negative**
TC	33 (38.8)	3 (3.5)	27 (31.8)	2 (2.3)	1 (1.2)	0 (0)
AC	7 (8.2)	0 (0)	7 (8.2)	0 (0)	0 (0)	0 (0)
SCLC	28 (33.0)	0 (0)	6 (7.1)	3 (3.5)	18 (21.2)	1 (1.2)
LCNLC	17 (20.0)	0 (0)	12 (14.1)	1 (1.2)	4 (4.7)	0 (0)
NEN all	85 (100)	3 (3.5)	52 (61.2)	6 (7.0)	23 (27.1)	1 (1.2)

Table 3. List of cases with at least one NE marker failing to prove the NE differentiation. Note that a panel of STX1 and INSM1 could clearly outperform the currently accepted CHGA, SYP and CD56 panel in the presented series.

Case #	**Diagnosis**	**STX1**	**INSM1**	**CGRA**	**SYP**	**CD56**
22	SCLC	Positive	Positive	Negative (<5%)	Positive	Positive
36	SCLC	Negative	Positive	Negative	Negative	Positive
53	SCLC	Positive	Positive	Positive	Negative	Positive
54	TC	Positive	Positive	Negative	Positive	Positive
56	LCNLC	Positive	Positive	Negative	Negative	Positive
60	SCLC	Positive	Positive	Negative	Negative	Positive
65	TC*****	Positive		Negative		
66	TC	Positive	Negative	Positive	Positive	Positive
72	TC	Positive	Negative	Positive	Negative (<5%)	Negative
75	SCLC	Positive	Positive	Negative	Negative	Negative (<5%)
77	LCNLC******	Positive	Positive	Negative	Negative (<5%)	Negative (<5%)
79	SCLC	Positive	Positive	Negative (<5%)	Negative (<5%)	Positive

* The SYP reaction produced for the original pathology report was positive; but the tissue cores were damaged, and only interpretable in the CHGA and STX1 stained TMA blocks.

**The SYP reaction produced for the original pathology report demonstrated SYP positivity (30%; 3+)

	CHGA		SYP		CD56		INSM1	
	Sens (%)	**Range**	**Sens (%)**	**Range**	**Sens (%)**	**Range**	**Sens (%)**	**Range**
TC	98.1	93.1-100.0	98.5	93.8/96.2-100.0	98.0	83.3-100.0	90.1	81.3-100.0
AC	96.4	92.9-100.0	98.2	86.7-100.0	93.7	80.0-100.0	87.6	74.3-100.0
SCLC	67.9	22.2-100.0	81.1	56.9-100.0	92.9	70.0-100.0	91.8	86.1-100.0
LCNLC	48.0	25.0-100.0	76.5	61.0-100.0	85.0	60.9-100.0	66.8	41.6-100.0
All	**75.1**		**86.6**		**92.7**		**85.6**	

Table 4. Calculated sensitivity of chromogranin A, Synaptophysin, CD56, INSM1 antibodies among lung neuroendocrine neoplasms based on literature review. (CHGA: chromogranin A, SYP: synaptophysin, INSM1: insulinoma-associated protein 1, TC: typical carcinoid, AC: atypical carcinoid, SCLC: small cell lung cancer, LCNLC: large cell neuroendocrine lung cancer)

Supplementary table 1 Primary antibodies and protocols applied for immunohistochemistry

Antibody	Clone	Manufacturer	Retrieval	Dilution
Chromogranin A	LK2H10 (Mouse monoclonal)	Cellmarque	pH 9.0	1:700
Synaptophysin	27G12 (Mouse monoclonal)	Novocastra	pH 9.0	1:400
CD56	123C3.D5 (Mouse monoclonal)	Cellmarque	pH 9.0	1:200
INSM1	A8 (Mouse monoclonal)	Santa Cruz	pH 9.0	1:100
STX1 (HPC-1)	sc-12736 (Mouse monoclonal)	Santa Cruz	pH 10.0	1:200

Supplementary Table 2 Publications evaluating CHGA, SYP, CD56 and INSM1 expression by immunohistochemistry in pulmonary neuroendocrine neoplasms (CHGA: chromogranin A, SYP: synaptophysin, INSM1: insulinoma-associated protein 1, TC: typical carcinoid, AC: atypical carcinoid, SCLC: small cell lung cancer, LCNLC: large cell neuroendocrine lung cancer)

Author (year)	cut off	Tumor type	CHGA positive/all	SYP positive/all	CD56 positive/all	INSM1 positive/all
Nicholson et al [30] (2002)	≥ 1%	SCLC	46/80	41/72	NA	NA
Yeh et al [31] (2014)	>10%	TC	27/29	25/26	20/24	NA
		AC	4/4	4/4	4/5	NA
		SCLC	12/35	25/35	32/33	NA
		LCNLC	7/17	12/17	17/17	NA
Rooper et al [4] (2017)	Any staining	TC	23/23	23/23	18/18	23/23
		AC	18/18	18/18	15/18	18/18
		SCLC	19/39	21/30	21/30	37/39
		LCNLC	11/23	14/14	14/23	21/23
Doxtader et al [32](2018)	any staining	SCLC	14/14	37/40	40/40	38/41
		LCNLC	1/1	1/1	1/1	1/1
Kriegsmann et al [33] (2018)	at least weak in ≥ 1% of cells	TC	111/112	111/112	112/112	91/112
		AC	39/39	39/39	39/39	29/39
		SCLC	107/144	122/144	132/144	124/144
		LCNLC	32/77	47/77	66/77	32/77
Mukhopadhyay et al [34] (2019)	any staining	TC	45/45	48/48	42/42	48/48
		AC	16/16	16/16	16/16	15/16
		SCLC	53/64	64/64	61/64	63/64
		LCNLC	11/24	21/24	22/24	18/24

Viswanathan et al [35] (2019)	2+	TC	11/11	11/11	11/11	11/11
		AC	11/11	11/11	10/11	11/11
		SCLC	2/9	7/9	9/9	8/9
		LCNLC	2/8	5/8	8/8	6/8
	≥ 1+ in > 5%	TC	17/17	17/17	17/17	16/17
		AC	13/14	13/15	12/14	12/14
		SCLC	3/8	8/8	8/8	8/8
		LCNLC	4/10	9/10	9/10	8/10
Staaf et al [36] (2020)	any staining	SCLC	16/24	20/24	23/24	22/24
		LCNLC	14/23	23/23	19/23	20/23

Supplementary table 3 Clinicopathological features of patients evaluated (TC: typical carcinoid, AC: atypical carcinoid, SCLC: small cell lung cancer, LCNLC: large cell neuroendocrine lung cancer, pT, pN stage: categories defined by AJCC, x: no data).

Age	**years**		**Lymphovascular invasion**	**n**	**%**
average	62.13		**L0/L1**	50/35	41.2
range	30-79.6		**Spread through airspaces**	**n**	**%**
			STAS0/STAS1	57/28	33.0
Surgery	**n**	**%**	**pT category**	**n**	**%**
sublobectomy	10	11.8	**pT1a**	34	40.0
lobectomy	75	88.2	**pT1b**	14	16.5
Localization	**n**	**%**	**pT1c**	10	11.8
right upper lobe	19	22.4	**pT2a**	15	17.6
right middle lobe	3	3.5	**pT2b**	8	9.4
right lower lobe	20	23.5	**pT3**	3	3.5
left upper lobe	24	28.2	**pT4**	1	1.2
left lower lobe	19	22.4	**pN category**	**n**	**%**
Subtype	**n**	**%**	**pN0**	52	61.2
TC	33	38.8	**pN1**	12	14.1
AC	7	8.2	**pN2**	18	21.2
SCLC	28	33.0	**pNx**	3	3.5
LCNLC	17	20.0	**Stage**	**n**	**%**
Complete resection	**n**	**%**	**I**	48	56.5
R0/R1	85/0	100	**II**	15	17.6
Vascular invasion	**n**	**%**	**III**	19	22.4
V0/V1	82/3	3.5	**x**	3	3.5

Figure legends:

Fig. 1 A: Typical carcinoid. Note the "salt and pepper" chromatin of the nuclei at high magnification. Prominent nucleoli, necrosis and mitosis are absent. (HE, 40x), B: Diffuse membranous STX1 expression with strong intensity in the same case as shown in A (STX1, 40x) C: Atypical carcinoid. It is similar to typical carcinoid, but punctate necrosis and/or elevated mitotic activity are present. Note the mitosis (arrow). (HE, 40x), D: Diffuse membranous STX1 expression in the same case as shown in C. Arrow points at a mitotic figure (STX1, 40x) E: Small cell lung cancer. The nuclei demonstrate the "salt and pepper" granulation. Geographical necrosis and high mitotic activity were present elsewhere. (HE, 40x), F: Diffuse cytoplasmic and membranous STX1 expression was identified in the same case as shown in E. Note the mitotic figure. (STX1, 40x) G: Large cell neuroendocrine lung cancer. Despite of the typical neuroendocrine nuclear features, vesicular chromatin and prominent nucleoli are present. Comedo-like necrosis and high mitotic activity were seen in other areas. Note the rosette formation and peripheral palisading. (HE, 40x), H: Diffuse cytoplasmic and membranous STX1 expression were seen in the same case as shown in G. (STX1, 40x)