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# NOL4 is a novel nuclear marker of small cell carcinoma and other neuroendocrine neoplasms

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**Summary.** Neuroendocrine neoplasms (NENs) such as small cell lung cancer (SCLC) and large cell neuroendocrine carcinoma (LCNEC) have characteristic histologies, but immunohistochemistry using neuroendocrine markers is still desirable to confirm diagnosis. CD56 is the most sensitive marker, but also stains various normal tissues and other tumors. Recently, we reported that nucleolar protein 4 (NOL4) is present in the blood of SCLC patients and found it was stained in the SCLC nuclei. In this study, we compared expressions of NOL4 and CD56, using 64 cases of SCLC, 18 cases of LCNEC, 6 cases of atypical carcinoid tumor, 7 cases of typical carcinoid tumor, 68 cases of lung adenocarcinoma, and 62 cases of lung squamous cell carcinoma. For primary lung NENs, sensitivity, specificity, positive predictive value (PPV) and negative predictive value of NOL4 were 77.5%, 95.8%, 93.2%, and 85.1%, respectively, while those of CD56 were 92.1%, 93.3%, 91.1%, and 94.1%. The specificity and PPV of NOL4 were higher than those of CD56, although the differences were not statistically significant. However, NOL4 retains its nuclear immunoreactivity in areas of crush artifact or necrosis. Furthermore, NOL4 was not expressed in adjacent normal tissues including bronchial cells and pneumocytes. Therefore, a combination of NOL4 staining with other cytoplasmic or membranous neuroendocrine markers might enhance diagnostic utility for SCLC and other NENs.

**Key words:** Neuroendocrine, NOL4, Small cell lung cancer, Biomarker

## Introduction

Neuroendocrine neoplasms (NENs) are characterized by the presence of neurosecretory granules with characteristic histologies and immunoprofiles (Rindi et al., 2018). NENs encompass both well-differentiated neuroendocrine tumors (NETs) and poorly differentiated neuroendocrine carcinomas (NECs) and occur throughout the entire body. Small cell lung cancer (SCLC) is one of the most common NENs, and the diagnosis of SCLC is critical because treatment and prognosis are totally different from those of other lung tumors (Takahashi et al., 1991; Varghese et al., 2014; Byers and Rudin, 2015; WHO, 2021).

The morphological recognition of classic cases of SCLC is straightforward for general pathologists, but immunohistochemistry to determine the presence of neuroendocrine differentiation is still worthwhile. Although the expression of neuroendocrine markers is not an essential diagnostic criteria for SCLC, it is highly desirable in the clinical context (WHO, 2021). Several antibodies are being used for this purpose, such as CD56, chromogranin A, and synaptophysin, but all have their strengths and weaknesses. CD56 (or NCAM, neural cell adhesion molecule) has high sensitivity for SCLC but shows positive staining in various normal tissues and other tumors, for example, it stains submucosal glands and nerve bundles in bronchi, non-neuroendocrine carcinomas, myeloid leukemias, some T cell lymphomas, rhabdomyosarcoma, and multiple myeloma (Shipley et al., 1997; Uccella et al., 2018). Chromogranin A and synaptophysin are both well-known neuroendocrine markers and have been regarded as true markers of neuroendocrine differentiation

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**Abbreviations.** INSM1, insulinoma-associated protein 1; LCNEC, large cell neuroendocrine carcinoma; NCAM, neural cell adhesion molecule; NEC, neuroendocrine carcinoma; NEN, neuroendocrine neoplasm; NET, neuroendocrine tumor; NOL4, nucleolar protein 4; NPV, negative predictive value; PPV, positive predictive value; SCLC, small cell lung cancer; TTF-1, thyroid transcription factor-1

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because they detect parts of neurosecretory granules and synaptic vesicles, but their sensitivities are limited and their staining intensities are frequently weak (Yatabe et al., 2019). Yatabe et al. summarized the median and range of sensitivity: for CD56, 97% (79%-100%); for chromogranin A, 47% (4%-58%); and for synaptophysin, 67% (57%-83%).

Recently, insulinoma-associated protein 1 (INSM1), a transcription factor expressed in nuclei, was introduced as a neuroendocrine marker, and it has been reported that its sensitivity and specificity are superior to CD56, synaptophysin, or chromogranin A (Rooper et al., 2017; Tsai et al., 2020). However, there was a difference between reported sensitivities and specificities of INSM1. It may derive from the different positivity thresholds used. In the study of Tsai et al. (2020), INSM1 was expressed in non-neuroendocrine thoracic tumors such as thymic carcinoma, diffuse large B cell lymphoma, and squamous cell carcinoma, which caused the authors to conclude weak focal INSM1 expression is insufficient for a diagnosis of SCLC.

Nucleolar protein 4 (NOL4) was initially known as nucleolar localized protein and was first entirely isolated from a human fetal brain cDNA library (Ueki et al., 1998). NOL4 has been considered as cancer-testis antigen as its cDNA was found in brain and testis. When Ueki et al. first isolated NOL4, they did not observe its expression in normal lung tissues by RT-PCR or Northern blot but derived its identical expressed sequence tags from an SCLC cell line (NCI-H69) (Ueki et al., 1998). We first isolated NOL4 from SCLC patients by SEREX (serological analysis of expression cDNA libraries) and found it was stained in the SCLC nuclei in a previous study (Kim et al., 2021). Thus, we speculated that NOL4 staining might be useful for diagnosing SCLC. The interpretation of nuclear staining may be easier than that of cytoplasmic staining, especially when only a few tumor cells are intermingled with necrosis and crush artifacts, which are frequently observed in SCLC. Therefore, it would appear a combination of NOL4 staining with other cytoplasmic or membranous neuroendocrine markers might enhance diagnostic utility for SCLC and other NENs.

The purpose of the current study was two-fold: (1) to investigate the expression of NOL4 in pulmonary NENs, namely, SCLC, large cell neuroendocrine carcinoma (LCNEC), atypical carcinoid tumor, and typical carcinoid tumor and (2) to determine whether NOL4 might be a universal neuroendocrine marker expressed in NENs of various organs such as lung, pancreas, stomach, and large intestine. To better evaluate sensitivity and specificity, we simultaneously compared the expressions of NOL4 and CD56.

## Materials and methods

### *Tissue samples*

This study was approved by the Institutional Review Board of Pusan National University Yangsan Hospital (PNUYH) (approval number: 05-2020-010). The archives of the Department of Pathology at PNUYH were searched for NENs of the lung from December 2008 to March 2017. Cases with a putative but negative diagnosis for NEN as determined by the expressions of well-known neuroendocrine markers (e.g., CD56 and synaptophysin) or with insufficient tissue quantity were excluded. A total of 95 consecutive cases were selected, which included SCLCs (n=64, 51 biopsies and 13 resections), LCNECs (n=18, 6 biopsies and 12 resections), atypical carcinoid tumors (n=6 resections), and typical carcinoid tumors (n=7, 2 biopsies and 5 resections). Primary lung adenocarcinoma (n=68) and squamous cell carcinoma (n=62) samples from resected specimens were obtained from a previous study as controls (Kim et al., 2015). NENs arising in organs other than lungs were also included in this study (n=108). The organs of NENs in other than lungs included 64 gastrointestinal (GI) tracts (4 biopsies and 60 resections), 14 pancreatobiliary systems (14 resections), 4 head and necks (1 biopsy and 3 resections), 3 genitourinary systems (1 biopsy and 2 resections), 12 livers (10 biopsies and 2 resections), and 11 lymph nodes (9 biopsies and 2 resections). Liver and lymph node lesions were all metastatic tumors. Human testis tissue was used as a positive control.

### *Immunohistochemistry*

Representative 4-μm sections of formalin-fixed paraffin-embedded tissues were cut on charged glass slides. Slides were placed in a 65°C oven for 20 min to dry tissues. Immunostaining was performed using a Bond-Max (Leica, Wetzlar, Germany) or a Ventana Benchmark ULTRA (Roche Diagnostics, Basel, Switzerland) staining instrument as described by the manufacturer. Briefly, paraffin wax was removed using Bond Dewax Solution (AR9222, Novocastra, Newcastle upon Tyne, UK) or EZ prep solution (Ventana Medical Systems, Mountain View, CA, USA) at 72°C and sections were rehydrated. Heat-induced epitope retrieval was achieved using Bond Epitope Retrieval Solution (citrate-based buffer) for 20 min or Ultra Cell Conditioning Solution 1 (Ventana Medical Systems, Mountain View, CA, USA) for 64 min. Sections were then incubated with monoclonal NOL4 (H00008715-M01, 1:200; Novus, Littleton, CO, USA; Bond-Max; 20 min), synaptophysin (27G12, 1:250; Novocastra, Newcastle upon Tyne, UK; Bond-Max; 15 min), and CD56 (MRQ-42, ready-to-use; Cell Marque, Rocklin, CA, USA; Ventana; 40 min) at room temperature. Finally, 3,3′-diaminobenzidine tetrahydrochloride was used as the chromogen.

### *Interpretation of reactivity*

Intensities and proportions of nuclear NOL4 immunostaining were assessed by two pathologists (LJH

and SDH) (Fig. 1). Immunohistochemical intensities were scored semi-quantitatively using a 4-point scale: 0 (no signal in any tumor cell); 1+ (weak signal under high magnification); 2+ (weak signal under low magnification); or 3+ (strong signal under low magnification). We defined NOL4 immunostaining as positive when the intensity was ≥2+ or the percentage of stained cells exceeded 50%.

For CD56, any cytoplasmic or membranous staining was considered positive. Any granular cytoplasmic staining was considered positive for synaptophysin.

### *Statistical analysis*

Statistical analysis was carried out using the software package R Project for Statistical Computing (version 4.0.5) (R Core Team, 2021). The chi-square test was used to compare categorical variables and the Mann-Whitney test to compare continuous variables. *p*-values of <0.05 were considered significant. The performance of NOL4 as a neuroendocrine tumor marker was compared with that of CD56 using an R program *compbdt*, which also generated sensitivities, specificities, likelihood ratios, and predictive values (Roldán-Nofuentes, 2020).

## Results

### *Clinical features of the primary lung cancer patients*

Male patients predominated in SCLC (57/64, 89%) and squamous cell carcinoma (59/62, 95%) cases,

**Table 1.** Summary of NOL4 staining in the primary lung tumors.

| | Total | NOL4-positive | NOL4-negative |
|---|---|---|---|
| NENs | | | |
| Small cell carcinomas | 64 | 53 (83%) | 11 (17%) |
| Large cell neuroendocrine carcinomas | 18 | 10 (56%) | 8 (44%) |
| Atypical carcinoid tumor | 6 | 5 (83%) | 1 (17%) |
| Typical carcinoid tumor | 7 | 6 (86%) | 1 (14%) |
| | 95 | 74 (78%) | 21 (22%) |
| Non-NENs | | | |
| Adenocarcinomas | 68 | 3 (4%) | 65 (96%) |
| Squamous cell carcinomas | 62 | 2 (3%) | 60 (97%) |
| | 130 | 5 (4%) | 125 (96%) |

NENs, neuroendocrine neoplasms.

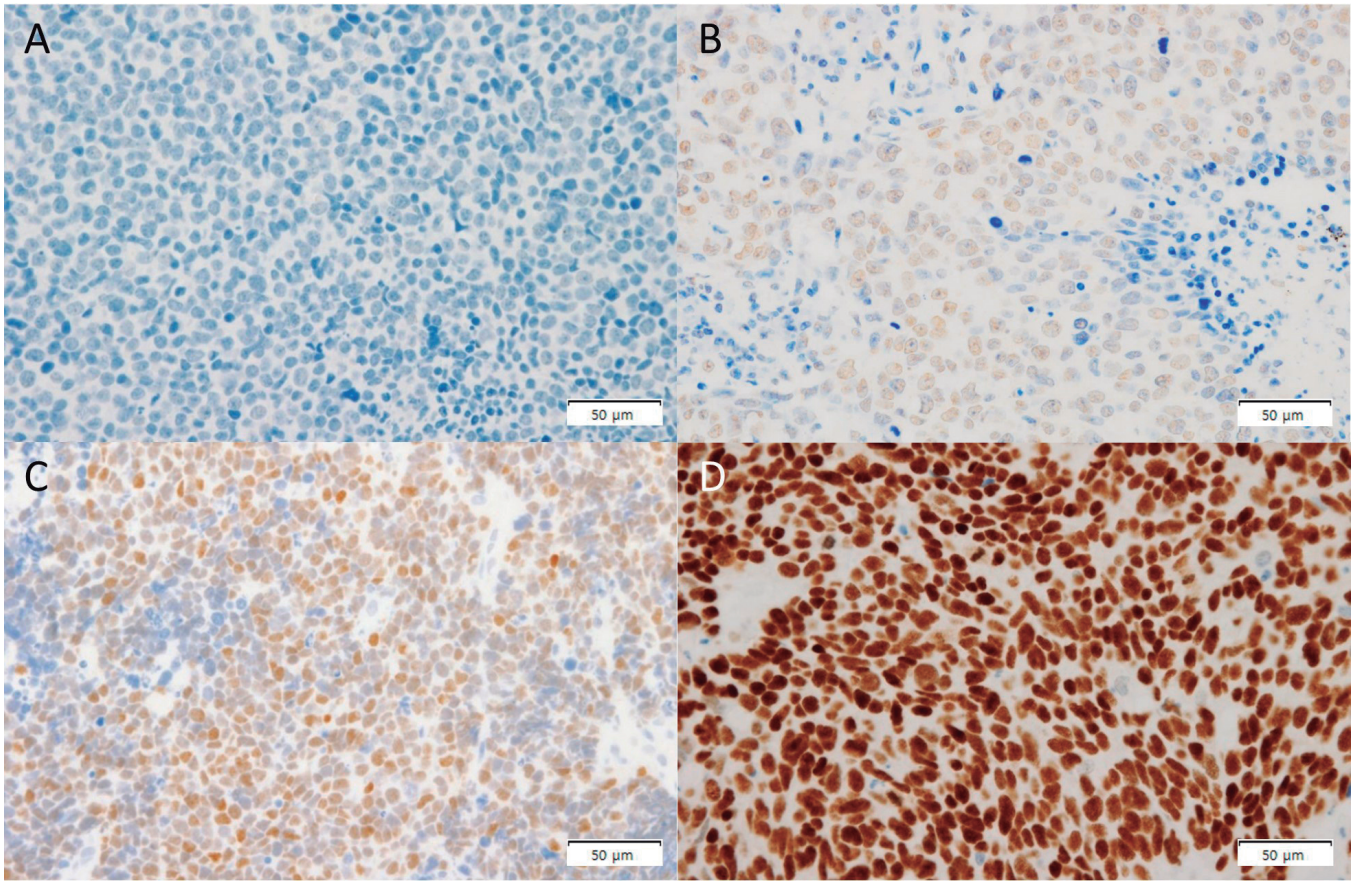


**Fig. 1.** The grade of intensity of NOL4 immunostaining in small cell lung cancer. **A.** Intensity 0. **B.** Intensity 1. **C.** Intensity 2. **D.** intensity 3. x 400.

whereas no significant sex difference was observed for adenocarcinomas (28 females vs. 40 males; $p$=0.39). Ages of SCLC patients ranged from 50 to 88 years (median, 70 y) and those of adenocarcinoma patients from 29 to 81 years (median, 65 y; $p$=0.04). Primary SCLC, adenocarcinoma, and squamous cell carcinoma lesions were distributed evenly across right and left lobes.

**Table 2.** Comparison of NOL4, CD56, and synaptophysin expression in the primary lung tumors.

| | NOL4 | CD56 | Synaptophysin |
|---|---|---|---|
| NENs | | | |
| Small cell carcinoma | 53/64 (83%) | 55/60 (92%) | 50/61 (82%) |
| Large cell neuroendocrine carcinoma | 10/18 (56%) | 15/16 (94%) | 7/11 (64%) |
| Atypical carcinoid tumor | 5/6 (83%) | 5/6 (83%) | 5/5 (100%) |
| Typical carcinoid tumor | 6/7 (86%) | 7/7 (100%) | 5/5 (100%) |
| Non-NENs | | | |
| Adenocarcinoma | 3/68 (4%) | 2/63 (3%) | - |
| Squamous cell carcinoma | 2/62 (3%) | 6/56 (11%) | 0/59 (0%) |

NENs, neuroendocrine neoplasms.

**Table 3.** Comparison of NOL4 and CD56 performance in diagnosing the primary neuroendocrine neoplasm (NEN) of the lung.

| | NOL4(+) | | NOL4(-) |
|---|---|---|---|
| | CD56(+) | CD56(-) | CD56(+) |
| NEN* | 68 | 1 | 14 |
| Non-NEN† | 2 | 3 | 6 |

| | NOL4 | CD56 | p-value |
|---|---|---|---|
| Sensitivity | 77.5% (67.9-85.0%) | 92.1% (84.8-96.3%) | < 0.001 |
| Specificity | 95.8% (90.6-98.3%) | 93.3% (87.4-96.7%) | 0.657 |
| Positive likelihood ratio | 18.5 (8.4-41.8) | 13.7 (7.4-25.4) | 0.533 |
| Negative likelihood ratio | 0.24 (0.16-0.34) | 0.08 (0.04 - 0.16) | 0.002 |
| Positive predictive value | 93.2% (85.3-97.2%) | 91.1% (83.6- 95.5%) | 0.531 |
| Negative predictive value | 85.1% (78.1-90.2%) | 94.1% (88.4-97.2%) | 0.001 |

*: Small cell carcinoma, Large cell neuroendocrine carcinoma, Typical carcinoid tumor, Atypical carcinoid tumor.
†: Adenocarcinoma, Squamous cell carcinoma.

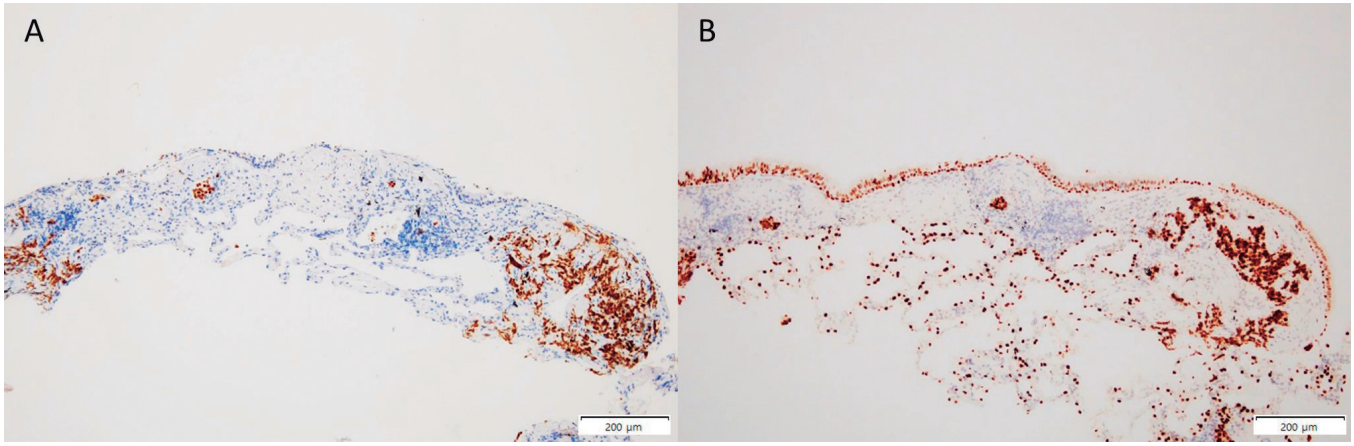


**Fig. 2.** As a nuclear marker for the pulmonary neuroendocrine neoplasms, NOL4 has a better performance in revealing tumor cells. **A.** NOL4 is expressed only in tumor cells of small cell lung cancer. **B.** TTF-1 is also expressed in normal pneumocytes and bronchial epithelial cells of the same tissue. Biopsy specimen, x 100.

### *NOL4 staining in non-neoplastic tissues*

In lung tissues, NOL4 was not expressed in pneumocytes, club cells, submucosal glands, or bronchial cells (Fig. 2). Rare pulmonary neuroendocrine cells were stained and neuroendocrine cells in stomach and pancreas were also stained. Nerve bundles and ganglion cells did not express NOL4 in any tissue.

**Table 4.** NOL4 staining in the non-pulmonary neuroendocrine neoplasms.

| Category | No. of cases | No. of NOL4-positive cases | Proportion |
|---|---|---|---|
| Gastrointestinal tract | | | 48/64 (75%) |
| Esophagus | 1 | 1 | |
| Stomach | 12 | 6 | |
| Duodenum | 10 | 6 | |
| Jejunum | 1 | 1 | |
| Ileum | 2 | 1 | |
| Appendix | 4 | 2 | |
| Colon | 7 | 5 | |
| Rectum | 27 | 26 | |
| Pancreatobiliary system | | | 11/14 (79%) |
| Pancreas | 10 | 7 | |
| Common bile duct | 1 | 1 | |
| Gallbladder | 3 | 3 | |
| Head and neck | | | 3/4 (75%) |
| Larynx | 1 | 1 | |
| Parotid gland | 1 | 1 | |
| Middle ear | 1 | 1 | |
| Nasal cavity | 1 | 0 | |
| Genitourinary system | | | 3/3 (100%) |
| Urinary bladder | 1 | 1 | |
| Uterine cervix | 1 | 1 | |
| Testis | 1 | 1 | |
| Liver (metastatic) | 12 | 8 | 8/12 (67%) |
| Lymph node (metastatic) | 11 | 9 | 9/11 (82%) |
| TOTAL | 108 | 82 | 82/108 (76%) |

### *NOL4 staining in the primary lung tumors*

As visualized in the expression pattern plots (Fig. 3), NOL4 exhibited more intense and more widespread immunoreactivity in SCLCs than in adenocarcinomas or squamous cell carcinomas, in which NOL4 was not expressed or expressed weakly.

NOL4 was expressed in 53 of 64 SCLCs (83%), 3 of 68 adenocarcinomas (4%), and 2 of 62 squamous cell carcinomas (3%) of the lung (Table 1). Carcinoid tumors, both atypical and typical, had NOL4 positivity rates similar to SCLCs (83% and 86%) and around half lung LCNECs (56%) were NOL4 positive.

### *NOL4 performance as a neuroendocrine tumor marker*

When the subjects were classified into non-NEN (adenocarcinomas and squamous cell carcinomas) and NEN (SCLCs, LCNECs, atypical carcinoid tumors, and typical carcinoid tumors) groups, NOL4 immunoreactivity was positive in 5 of 130 in the non-NEN Group (4%) and in 74 of 95 in the NEN Group (78%), in which NOL4 immunoreactivity showed a sensitivity of 78%, a specificity of 96%, a positive predictive value (PPV) of 94%, and a negative predictive value (NPV) of 86% (calculated from Table 1).

In pulmonary NENs, the positive reaction rate of NOL4 staining was lower than that of CD56 and synaptophysin staining (Table 2). However, NOL4 expression tended to be more specific and positively

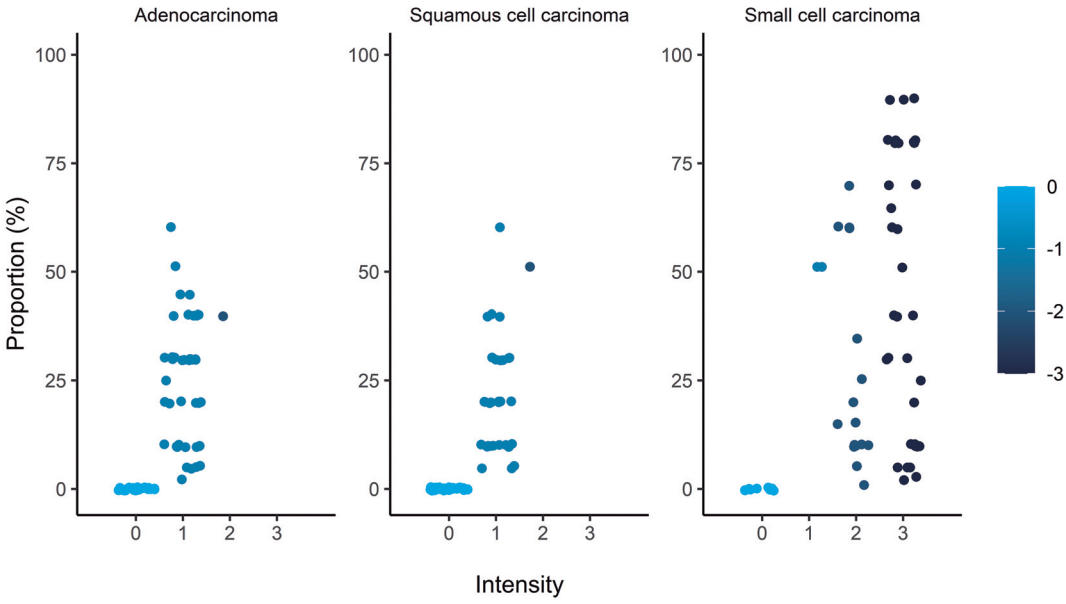


**Fig. 3.** Combined scatter plot of the proportion and intensity of NOL4 expression in adenocarcinomas, squamous cell carcinomas, and small cell carcinomas of the lung (the points in the plot were jittered in order to avoid being piled up on top of one another).

predictive than CD56 expression (Table 3). Furthermore, negative likelihood ratio of NOL4 was higher than that of CD56 (p=0.002). On the other hand, CD56 had a higher sensitivity (p<0.001) and more negative predictive value (p=0.001) than NOL4.

### *NOL4 staining in the non-pulmonary neuroendocrine neoplasms*

We also compared NOL4 reactivities in 108 cases of non-pulmonary NENs (Table 4). GI tract, pancreato-biliary system, and head and neck cases had similar positivity rates (75%, 79%, and 75%, respectively). Although the number of cases was limited (n=3), all genitourinary system cases exhibited NOL4 reactivity.

Non-pulmonary cases included 23 metastatic lesions to liver and lymph nodes. Metastatic NENs stained positively for NOL4 in 67% of hepatic and 82% of nodal lesions.

## Discussion

A diagnosis of SCLC can be made with near certainty based on characteristic morphologic findings in hematoxylin and eosin staining, and is subsequently confirmed by immunostaining for neuroendocrine markers including CD56, synaptophysin, and chromogranin A. CD56 is the most widely used neuroendocrine marker because of its high sensitivity. Other markers are less sensitive, and thus are utilized as supplementary markers. However, when CD56 is negative for tumors morphologically suspected as being SCLC, another marker is helpful to confirm the diagnosis. Recently, we reported that NOL4 mRNA is present in the blood of SCLC patients (Kim et al., 2021), and in the present study, NOL4 was expressed in SCLC and other pulmonary NENs. In addition, a subsidiary evaluation was also made to determine whether NOL4 is expressed in NENs of other organs.

In primary lung tumors, the sensitivity, specificity, PPV and NPV of NOL4 for NENs were 77.5%, 95.8%, 93.2%, and 85.1%, respectively, while those of CD56 were 92.1%, 93.3%, 91.1%, and 94.1% (Table 3). Thus, NOL4 was not superior to CD56 in terms of sensitivity and NPV, which may have been partly because tissue samples were collected from NEN cases verified using CD56. Meanwhile, the specificity and PPV of NOL4 were higher than that of CD56, although the difference

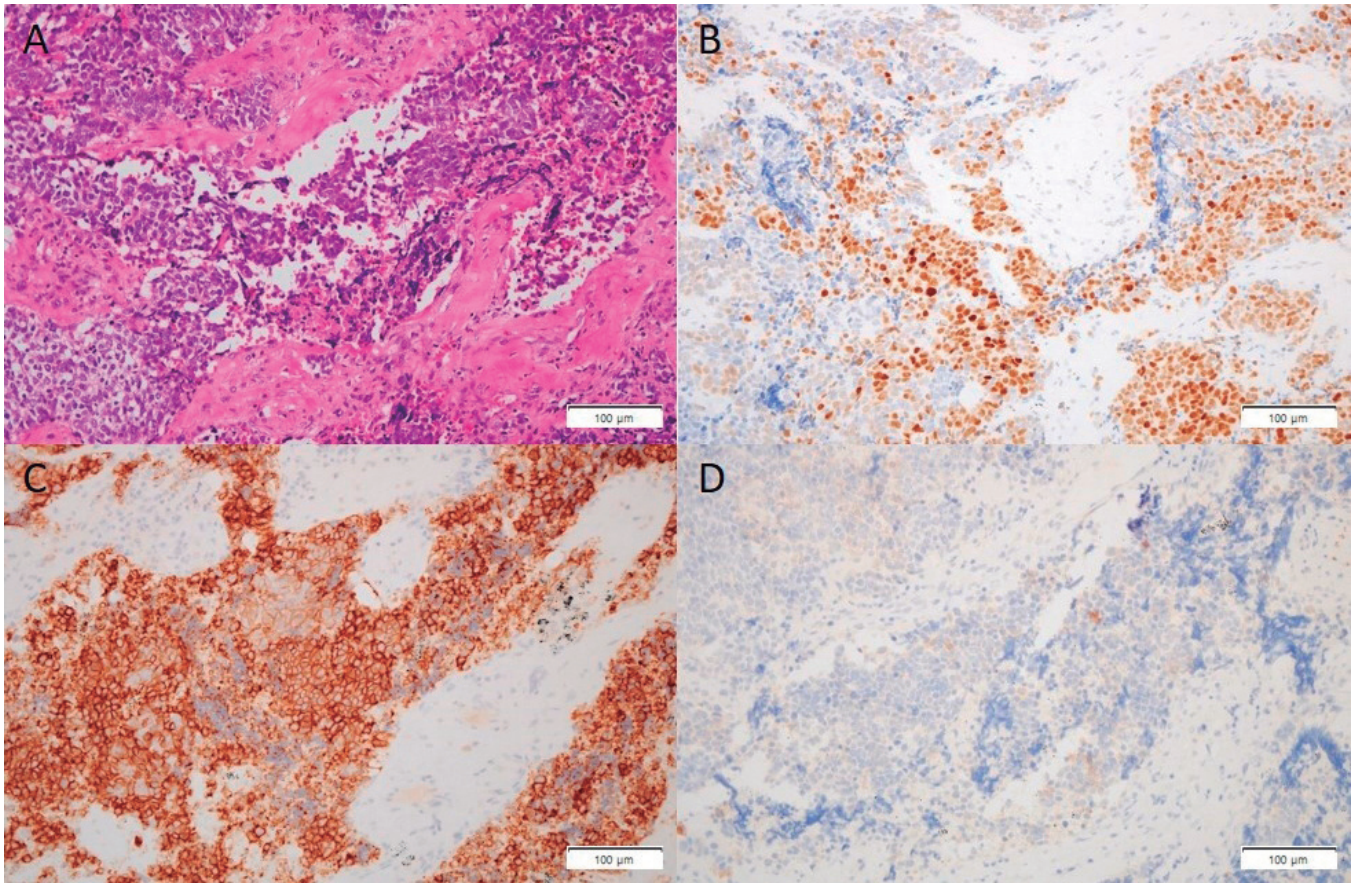


**Fig. 4. A.** Small cell lung cancer shows crush artifact and necrosis (Hematoxylin and Eosin). **B.** NOL4 is expressed in the nucleus of tumor cells. **C.** CD56 shows membranous staining. **D.** Expression of synaptophysin is faint in tumor cells. x 200.

was not statistically significant. However, NOL4 has specific advantages. First, NOL4 retains its immunoreactivity in areas of crush artifact or necrosis (Fig. 4A,B). Other cytoplasmic and membranous markers are faintly expressed in these areas because the antigen is lost during the process of crushing or necrosis (Fig. 4D). However, nuclear antigens may be better preserved under these circumstances. Second, interpretation is more straightforward for NOL4. As a nuclear stain, it generally showed less background staining and better resolution. The intensity of NOL4 expression was at least moderate in most SCLCs, although the proportion of cells expressing NOL4 were varied. Thus, it might have been reasonable to define NOL4 expression as positive when the intensity, as determined using our scale, was stronger than or equal to 2+ or the percentage of NOL4-expressing cells was higher than 50%. Third, NOL4 was not expressed in various normal lung tissues whereas other neuroendocrine markers are expressed in nerve fibers or neurons, which are present in bronchi and lung parenchyma. NOL4 was not expressed in neuronal tissues, bronchial cells, pneumocytes, or macrophages, and only in a few rare cells in bronchi, which were thought to be neuroendocrine cells. Although the benefit of a reliable internal positive control cannot be expected, the exclusive expression is much more important for diagnosis when dealing with small crushed tissue samples. Notably, our findings suggest that any aggregates of NOL4-expressing cells in lung should raise a high level of suspicion for NEN, especially in small biopsy specimens.

NOL4 was also found to be expressed in 85% of pulmonary carcinoid tumors and 56% of LCNECs. Furthermore, typical and atypical carcinoid tumors demonstrated positivity rates for NOL4 similar to SCLCs. In terms of proportions and intensities of NOL4 expression, carcinoid tumors and SCLCs were alike, except that carcinoid tumors showed more diffuse staining. On the other hand, LCNECs were positive for NOL4 in only 56% of cases, which suggests LCNECs could be divided into NOL4-positive and -negative groups. This observation is in line with recent studies, which revealed that pulmonary LCNECs are a biologically heterogeneous group of tumors, consisting of genetically distinct subsets, SCLC-like and non-SCLC-like (Rekhtman et al., 2016; George et al., 2018). Thus, NOL4-positive LCNEC possibly belongs to the SCLC-like subset and NOL4-negative LCNEC to the non-SCLC-like subset. Further research is required on the topic. The identification of SCLC-like LCNECs would be important because therapeutic responsiveness to SCLC regimens has been reported in some LCNEC series (Rossi et al., 2005).

As regards extrapulmonary NENs, the sensitivity of NOL4 was 75% for GI tumors and 79% for pancreatobiliary tumors. Notably, NOL4 was positive in 26 of 27 rectal NENs, and when rectal NENs were excluded from GI tract NENs, its sensitivity dropped to 59.5%. Therefore, the role of NOL4 as a universal neuroendocrine marker seems non-feasible for extrapulmonary NENs. Nonetheless, stain qualities were excellent without any background signals, and thus NOL4 can be a suitable complementary marker. For normal tissues, the nuclear expression of NOL4 was observed in pancreatic tissues and gastric neuroendocrine cells.

NOL4 mRNA expression was first demonstrated in fetal brain and adult brain and testes by Ueki (Ueki et al., 1998). Intriguingly, these three tissues, especially fetal brain tissues, can be viewed as neuroendocrine systems (Ugrumov, 2010), which supports the notion that NOL4 is a neuroendocrine marker. Several authors have described the relationship between NOL4 and human cancer since Ueki suggested NOL4 might be involved in neuroendocrine tumorigenesis (Wang et al., 2008; Demokan et al., 2014; Sheikholeslami et al., 2020). In these studies, it was found that NOL4 gene promoter was frequently hypermethylated and epigenetically contributed to squamous cell carcinomas of the uterine cervix and head and neck and to papillary thyroid carcinomas. However, the baseline expressions of NOL4 protein in normal tissues of the uterine cervix, head and neck, or thyroid gland were not examined. Furthermore, comparisons of protein expressions in normal and tumor tissues should be undertaken to establish the tumorigenic effect of gene hypermethylation. Therefore, the hypothesis that the NOL4 gene acts as a tumor suppressor should be more meticulously investigated, and the methylation profile of NOL4 promoter in NENs also deserves study.

A study on the comparative performances of NOL4 and INSM1 is also required. INSM1 has been recently accepted as a neuroendocrine marker that stains the nucleus of SCLC. Since its diagnostic value was evaluated, its prognostic and therapeutic use have been actively explored. NOL4 also needs to be assessed from various perspectives and this study would be the first step toward this effort.

Summarizing, NOL4 is not a universal neuroendocrine marker but a qualitatively excellent and quantitatively reasonable neuroendocrine marker. For SCLCs, carcinoid tumors of lung, and carcinoid tumors of rectum, NOL4 was found to be almost as sensitive and specific as CD56. We believe its roles in cancer have yet to be discovered and that further studies should be conducted to establish the roles played by NOL4 in tumorigenesis and progression.

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*Conflicts of Interest.* The authors have no conflict of interest to declare.

## References

Byers L.A. and Rudin C.M. (2015). Small cell lung cancer: Where do we go from here? Cancer 121, 664-672.

Demokan S., Chuang A.Y., Pattani K.M., Sidransky D., Koch W. and Califano J.A. (2014). Validation of nucleolar protein 4 as a novel methylated tumor suppressor gene in head and neck cancer. Oncol. Rep. 31, 1014-1020.

George J., Walter V., Peifer M., Alexandrov L.B., Seidel D., Leenders F., Maas L., Müller C., Dahmen I., Delhomme T.M., Ardin M., Leblay N., Byrnes G., Sun R., De Reynies A., McLeer-Florin A., Bosco G., Malchers F., Menon R., Altmüller J., Becker C., Nürnberg P., Achter V., Lang U., Schneider P.M., Bogus M., Soloway M.G., Wilkerson M.D., Cun Y., McKay J.D., Moro-Sibilot D., Brambilla C.G., Lantuejoul S., Lemaitre N., Soltermann A., Weder W., Tischler V., Brustugun O.T., Lund-Iversen M., Helland Å., Solberg S., Ansén S., Wright G., Solomon B., Roz L., Pastorino U., Petersen I., Clement J.H., Sänger J., Wolf J., Vingron M., Zander T., Perner S., Travis W.D., Haas S.A., Olivier M., Foll M., Büttner R., Hayes D.N., Brambilla E., Fernandez-Cuesta L. and Thomas R.K (2018). Integrative genomic profiling of large-cell neuroendocrine carcinomas reveals distinct subtypes of high-grade neuroendocrine lung tumors. Nat. Commun. 9, 1-13.

Kim M.H., Kim Y.K., Shin D.H., Lee H.J., Shin N., Kim A., Lee J.H., Choi K.U., Kim J.Y., Lee C.H. and Sol M.Y. (2015). Yes associated protein is a poor prognostic factor in well-differentiated lung adenocarcinoma. Int. J. Clin. Exp. Pathol. 8, 15933-15939.

Kim Y.R., Kim K.U., Lee J.H., Kim D.W., Chung J.H., Kim Y.D., Shin D.H., Lee M.K., Shin Y.I. and Lee S.Y. (2021). Cancer testis antigen, NOL4, is an immunogenic antigen specifically expressed in small-cell lung cancer. Curr. Oncol. 28, 1927-1937.

R Core Team. (2021). R: A language and environment for statistical computing. Vienna, Austria.

Rekhtman N., Pietanza M.C., Hellmann M.D., Naidoo J., Arora A., Won H., Halpenny D.F., Wang H., Tian S.K., Litvak A.M., Paik P.K., Drilon A.E., Socci N., Poirier J.T., Shen R., Berger M.F., Moreira A.L., Travis W.D., Rudin C.M. and Ladanyi M. (2016). Next-generation sequencing of pulmonary large cell neuroendocrine carcinoma reveals small cell carcinoma-like and non-small cell carcinoma-like subsets. Clin. Cancer Res. 22, 3618-3629.

Rindi G., Klimstra D.S., Abedi-Ardekani B., Asa S.L., Bosman F.T., Brambilla E., Busam K.J., de Krijger R.R., Dietel M., El-Naggar A.K., Fernandez-Cuesta L., Klöppel G., McCluggage W.G., Moch H., Ohgaki H., Rakha E.A., Reed N.S., Rous B.A., Sasano H., Scarpa A., Scoazec J.Y., Travis W.D., Tallini G., Trouillas J., van Krieken J.H. and Cree I.A. (2018). A common classification framework for neuroendocrine neoplasms: An International Agency for Research on Cancer (IARC) and World Health Organization (WHO) expert consensus proposal. Mod. Pathol. 31, 1770-1786.

Roldán-Nofuentes J.A. (2020). Compbdt: An R program to compare two binary diagnostic tests subject to a paired design. BMC Med. Res. Methodol. 20, 1-11.

Rooper L.M., Sharma R., Li Q.K., Illei P.B. and Westra W.H. (2017). 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. 41, 1561-1569.

Rossi G., Cavazza A., Marchioni A., Longo L., Migaldi M., Sartori G., Bigiani N., Schirosi L., Casali C., Morandi U., Facciolongo N., Maiorana A., Bavieri M., Fabbri L.M. and Brambilla E. (2005). Role of chemotherapy and the receptor tyrosine kinases Kit, PDGFRα, PDGFRβ, and MET in large-cell neuroendocrine carcinoma of the lung. J. Clin. Oncol. 23, 8774-8785.

Sheikholeslami S., Azizi F., Ghasemi A., Alibakhshi A., Parsa H., Tavangar S.M., Shivaee S., Yeganeh M.Z., Hedayati M. and Teimoori-Toolabi L. (2020). *NOL4* is downregulated and hypermethylated in papillary thyroid carcinoma suggesting its role as a tumor suppressor gene. Int. J. Endocrinol. Metab. 18, e108510.

Shipley W., Hammer R., Lennington W. and Macon W. (1997). Paraffin immunohistochemical detection of CD56, a useful marker for neural cell adhesion molecule (NCAM), in normal and neoplastic fixed tissues. Appl. Immunohistochem. 5, 87-93.

Takahashi T., Suzuki H., Hida T., Sekido Y., Ariyoshi Y. and Ueda R. (1991). The p53 gene is very frequently mutated in small-cell lung cancer with a distinct nucleotide substitution pattern. Oncogene 6, 1775-1778.

Tsai H.K., Hornick J.L. and Vivero M. (2020). INSM1 expression in a subset of thoracic malignancies and small round cell tumors: Rare potential pitfalls for small cell carcinoma. Mod. Pathol. 33, 1571-1580.

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

Ueki N., Kondo M., Seki N., Yano K., Oda T., Masuho Y. and Muramatsu M.A. (1998). NOLP: Identification of a novel human nucleolar protein and determination of sequence requirements for its nucleolar localization. Biochem. Biophys. Res. Commun. 252, 97-102.

Ugrumov M. (2010). Developing brain as an endocrine organ: A paradoxical reality. Neurochem. Res. 35, 837-850.

Varghese A.M., Zakowski M.F., Helena A.Y., Won H.H., Riely G.J., Krug L.M., Kris M.G., Rekhtman N., Ladanyi M., Wang L., Berger M.F. and Pietanza M.C. (2014). Small-cell lung cancers in patients who never smoked cigarettes. J. Thorac. Oncol. 9, 892-896.

Wang S.S., Smiraglia D.J., Wu Y.-Z., Ghosh S., Rader J.S., Cho K.R., Bonfiglio T.A., Nayar R., Plass C. and Sherman M.E. (2008). Identification of novel methylation markers in cervical cancer using restriction landmark genomic scanning. Cancer Res. 68, 2489-2497.

WHO Classification of Tumours Editorial Board. (2021). Thoracic tumours. 5th ed. International Agency for Research on Cancer. Lyon (France). pp 133-149.

Yatabe Y., Dacic S., Borczuk A.C., Warth A., Russell P.A., Lantuejoul S., Beasley M.B., Thunnissen E., Pelosi G., Rekhtman N., Bubendorf L., Mino-Kenudson M., Yoshida A., Geisinger K.R., Noguchi M., Chirieac L.R., Bolting J., Chung J.H., Chou T.Y., Chen G., Poleri C., Lopez-Rios F., Papotti M., Sholl L.M., Roden A.C., Travis W.D., Hirsch F.R., Kerr K.M., Tsao M.S., Nicholson A.G., Wistuba I. and Moreira A.L. (2019). Best practices recommendations for diagnostic immunohistochemistry in lung cancer. J. Thorac. Oncol. 14, 377-407.

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