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Original Article

Immunosensitivity and specificity of insulinoma-associated protein 1 (INSM1) for neuroendocrine neoplasms of the uterine cervix

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ABSTRACT

Objective: Previously, we reported that insulinoma-associated protein 1 (INSM1) immunohistochemistry (IHC) showed high sensitivity for neuroendocrine carcinoma of the uterine cervix and was an effective method for histopathological diagnosis, but that its specificity remained to be verified. Therefore, the aim was to verify the specificity of INSM1 IHC for a large number of non-neuroendocrine neoplasia (NEN) of the cervix.
Methods: RNA sequences were performed for cell lines of small cell carcinoma (TCYIK), squamous cell carcinoma (SiHa), and adenocarcinoma (HeLa). A total of 104 cases of formalin-fixed and paraffin-embedded specimens, 16 cases of cervical NEN and 88 cases of cervical non-NEN, were evaluated immunohistochemically for conventional neuroendocrine markers and INSM1. All processes without antigen retrieval were performed by an automated IHC system.
Results: The transcripts per million levels of INSM1 in RNA sequences were 1505 in TCYIK, 0 in SiHa, and HeLa. INSM1 immunoreactivity was shown only in the TCYIK. Immunohistochemical results showed that 15 cases of cervical NEN showed positive for INSM1; the positivity score of the tumor cell population and the stain strength for INSM1 were high. Two of the 88 cases of cervical non-NENs were positive for INSM1 in one case each of typical adenocarcinoma and squamous cell carcinoma. The sensitivity of INSM1 for cervical NEN was 94%; specificity, 98%; the positive predictive value, 88%; and the negative predictive value, 99%.
Conclusion: INSM1 is an adjunctive diagnostic method with excellent specificity and sensitivity for diagnosing cervical NEN. Higher specificity can be obtained if morphological evaluation is also performed.

Keywords: Neuroendocrine Carcinoma; Insulinoma Associated Protein 1; INSM1; Cervical Cancer; Endometrial Cancer; Immunohistochemistry

Synopsis

The aim was to verify the specificity of insulinoma-associated protein 1 (INSM1) immunohistochemistry for 88 cases of non-neuroendocrine neoplasms and 16 cases of neuroendocrine neoplasms of the uterine cervix. INSM1 is an adjunctive diagnostic method with excellent specificity (98%) and sensitivity (94%) for diagnosing cervical neuroendocrine neoplasms.

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Conflict of Interest
No potential conflict of interest relevant to this article was reported.

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INTRODUCTION

Neuroendocrine carcinoma (NEC) of the uterine cervix is a rare disease that represents 1% to 5% of cervical cancers [1,2]. Compared with other histologic types of cervical cancer, cervical NEC is highly malignant; it gives rise to hematogenous metastases from an early stage and has a poor prognosis [3-5]. The 5-year survival rate is approximately 90% for International Federation of Gynecology and Obstetrics (FIGO) stage IB1 ordinary cervical cancers, but it has been estimated to be only 55% to 63% for FIGO stage IB1 cervical NEC [3-7]. Since the biological characteristics of cervical NEN differ from those of other histological types of cervical cancer, the therapeutic strategy must also differ. Systemic chemotherapy even in the early stages has been suggested as necessary to achieve complete control of a local cervical NEN lesion [3,6]. The National Comprehensive Cancer Center Network Clinical Practice Guidelines in Oncology (NCCN guideline) showed specific therapeutic strategies for cervical small cell neuroendocrine carcinoma (SCNEC) [8, 9].

Insulinoma-associated protein 1 (INSM1), a zinc-finger transcription factor related to neuroendocrine differentiation, is frequently expressed in neuroendocrine tumors. In a previous study, we showed that immunohistochemistry (IHC) is more sensitive for INSM1 than for synaptophysin (Syn), chromogranin A (CGA), and neural cell adhesion molecule (NCAM), and that INSM1 may therefore be useful as a neuroendocrine marker [10]. In cervical NEC, we showed that sensitivity was 95%, and that positivity for INSM1 matched histological findings [10].

Occasionally cervical NEC, in particular SCNEC shows a superficial resemblance to poorly differentiated or basaloid type of squamous cell carcinoma, necessitating immunohistochemical study. Although INSM1 has been considered to be a promising marker for neuroendocrine differentiation, a subset of squamous cell carcinoma in some organ can be focally positive for INSM1 and thus its specificity and cut-off point remains a matter. It was difficult to evaluate specificity in previous study, because we performed INSM1 IHC by a manual procedure in accordance with the approach used in that work. Therefore, in this study, the specificity and sensitivity of INSM1 for uterine cervical neuroendocrine neoplasia (NEN) were examined under stable staining conditions with an automated IHC system.

MATERIALS AND METHODS

1. Case selection

The study included patients diagnosed with FIGO (2018) [11] stage IB1 to IVB cervical NEN from 2004 to 2020 at St. Marianna University Hospital, Kanagawa, Japan, and Kumamoto University Hospital, Kumamoto, Japan. Two cases in Kumamoto University were reported previously as a case report [12]. In addition, we included cases evaluated as FIGO stage IB1 to IVB cervical non-NEC at St. Marianna University Hospital. All specimens for histological analysis were obtained before any chemotherapy or radiotherapy was performed.

The study was approved first by the ethics committee of St. Marianna University Hospital (Kanagawa, Japan) (approved No. 5138) and then by the ethics committee of Kumamoto University Hospital (approved No. 2032).

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2. Cell culture

HeLa and SiHa cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). The TCYIK cell line was obtained from RIKEN BRC through the National BioResource Project of MEXT/AMED, Japan. SiHa and HeLa cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin. TCYIK cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. All cell lines were grown at 37°C in a humidified atmosphere containing 5% CO_2.

3. RNA extraction and RNA-Seq

RNA was extracted from HeLa, SiHa, and TCYIK cells using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The Agilent Bioanalyzer 2100 system (Agilent Technology) was used to check RNA integrity (RIN). Total RNA (1 μg) was processed using NEBNext® Poly(A) mRNA Magnetic Isolation Module (NEB E7490), and 3 libraries were prepared with the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB E7530) following the manufacturer's instructions. DNA libraries were sequenced using Illumina NovaSeq 6000 to generate paired-end reads.

4. Preparation of hematoxylin and eosin (H&E)-stained sections and immunohistochemically stained sections

Four-μm-thick, formalin-fixed, paraffin-embedded (FFPE) sections were deparaffinized in xylene and rehydrated in a descending ethanol series. For the histologic re-evaluation of all cases, slides stained with H&E were used.

The sources of the antibodies to the 4 neuroendocrine markers used were as follows: INSM1, sc-271408, mouse monoclonal (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Syn, 27G12, mouse monoclonal (Nichirei Bioscience, Tokyo, Japan); CGA, DAK-A3, mouse monoclonal (DAKO, Santa Clara, CA, USA); and NCAM, CD56, mouse monoclonal (Leica Biosystems, Wetzlar, Germany). The optimal dilution of INSM1 antibody was 1:250. Antigens were retrieved by heat treatment in 0.01-M citrate buffer (pH 6.0) in a 95°C water bath for 40 minutes. Sections were reacted with diluted primary antibodies for 60 minutes and with secondary antibody (Histofine Simple Stain MAX-PO [MULTI], NICHIREI) for 30 minutes and visualized using DAB (DAKO, K3468, Liquid DAB and Substate Chromogen System) for 1 minute. Finally, the sections were counterstained with Mayer's hematoxylin. All processes without antigen retrieval were performed by an automated IHC system (HISTOSTAINER 48A; Nichirei Biosciences, Tokyo, Japan).

Syn, CGA, and NCAM (HISTOSTAINER 48A; Nichirei Biosciences) have been traditionally and routinely used as neuroendocrine markers at our institution.

Cell lines were centrifuged, formalin fixed, and FFPE specimens were prepared and performed IHC as same method of NEN sections.

5. Histological analysis

NEN and other histological diagnoses of the cervix were defined according to the 2020 World Health Organization (WHO) criteria [13]. NEN includes neuroendocrine tumors and NEC. Neuroendocrine tumor can be categorized as neuroendocrine tumor, grade 1 (NET G1) and grade 2 (NET G2). NEC can be categorized as SCNEC and large cell neuroendocrine carcinoma (LCNEC), respectively. Tumors admixed with NEC were defined as either SCNEC or LCNEC with components of adenocarcinoma or squamous cell carcinoma.

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6. Evaluation of histological and immunohistochemical findings

Histological and immunohistochemical findings were independently evaluated by pathologists (A.E., F.K., and J.K.). Immunohistochemical scoring was based on the proportion and strength of staining of positively stained cells. The amount of positive staining of the tumor cell population was scored depending on the proportion of positive cells in the tumor area, as follows: score 0 (0%), 1 (≤5%), 2 (6%–50%), and 3 (≥51%). Admixed tumors were evaluated in the area of the histological neuroendocrine tumor, whereby the non-neuroendocrine area was also evaluated. The cells with the highest stain strength were evaluated with a ×20 microscope objective and classified as grade 0 to grade 3+, as follows: grade 0, negative; 1+, weak (nuclear staining can barely be determined with a ×20 objective); 2+, medium (slightly weak staining in the nucleus at objective ×20, but enough to be easily determined); and 3+, strong (**Table S1**, **Fig. S1**).

RESULTS

1. Cell line

The principal component analysis (PCA) scatter plot and heatmap of TCYIK, HiHa, and HeLa cell lines showed that they were completely different in RNA sequences (**Fig. S2**). The transcripts per million (TPM) levels of INSM1 in RNA sequences were 1505 in TCYIK, 0 in SiHa, and 0 in HeLa (**Fig. S3**). INSM1 immunoreactivity was shown only in the TCYIK cell line; none was shown in the SiHa and HeLa cell line (**Fig. 1**).

2. Immunohistochemical results for cases

Sixteen cases of cervical NEN were analyzed, as follows: SCNEC (n=8); LCNEC (n=5); SCNEC and LCNEC combined (n=1); and NET G2 (n=2). Six of these 16 cases were admixed with adenocarcinoma or squamous cell carcinoma. The clinical characteristics and histological subtypes are shown in **Table 1** and **Table S2**. In addition, 88 cases of non-NEN of the uterine cervix were analyzed, as follows: squamous cell carcinoma, 61 cases; adenocarcinoma, 23 cases; adenosquamous carcinoma, 2 cases; and cervical intraepithelial neoplasia, grade 3, 2 cases. Ten of 16 cases were obtained from radical hysterectomy, three were obtained from cervical conization, and three were biopsy specimens.

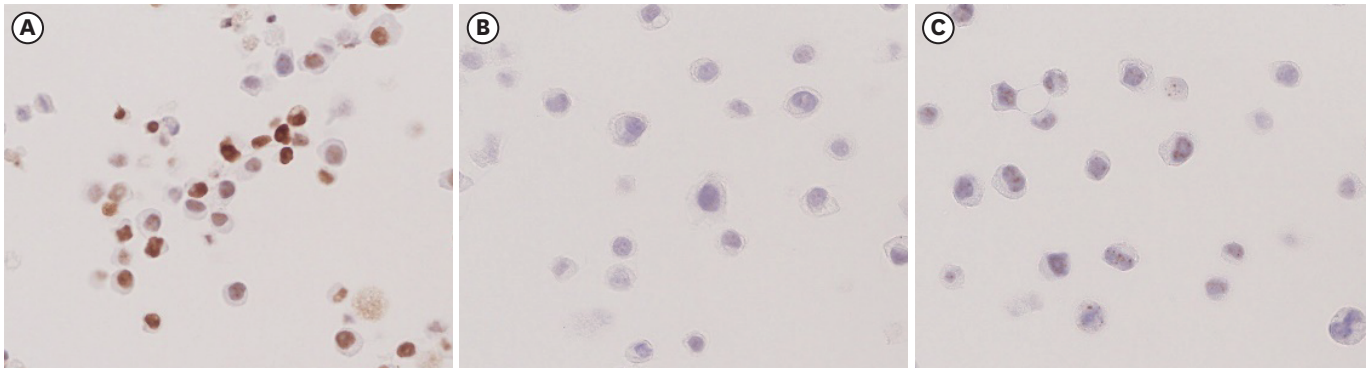

Fig. 1. INSM1 immunoreactivity for cell lines.
(A) TCYIK, (B) SiHa, and (C) Hela. INSM1 immunoreactivity was shown only in the TCYIK cell line.
INSM1, insulinoma-associated protein 1.

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Table 1. Types of tumors identified in patients diagnosed cervical neuroendocrine neoplasms and non-neuroendocrine neoplasia (n=104)

Types of tumors	Cases
Neuroendocrine neoplasia of the uterine cervix (n=16)	
SCNEC, pure	5 cases
SCNEC, admixed	3 cases
LCNEC, pure	2 cases
LCNEC, admixed	3 cases
SCNEC and LCNEC	1 case
NET G2	2 cases
Non-neuroendocrine neoplasia of the uterine cervix (n=88)	
Squamous cell carcinoma	61 cases
Adenocarcinoma, usual type	23 cases
Adenosquamous cell carcinoma	2 cases
Cervical intraepithelial neoplasia, grade 3	2 cases

NET G2, neuroendocrine tumor, grade 2; SCNEC, small cell neuroendocrine carcinoma; LCNEC, large cell neuroendocrine carcinoma.

INSM1 immunoreactivity was confined to the nuclei of positive cells. The cases of SCNEC and NET G2 with a tumor cell population positivity score of 3 and grade 3+ stain strength were shown in **Figure 2**; little background staining was seen, i.e., neither cytoplasmic nor membranous staining, and almost no staining of the nucleus or cytoplasm of non-neoplastic stromal cells was seen. All 16 cases (100%) of cervical NEN had tumor cells positive for INSM1. The tumor cell population positivity scores for INSM1 were as follows: score 3, 9 cases (56%); score 2, 5 cases (31%); score 1, 2 cases (13%); and score 0, 0 cases (0%) (**Table 2**). Of the 16 cases, 12 had grade 3+ stain strength, and 3 case had grade 2+. Only one case had grade 1+.

Two cases had score 1 and grade 3+ for INSM1, and the positive tumor cells were still easily recognizable (**Fig. 3**). In one of these cases, even though H&E staining identified a typical SCNEC, no staining was seen for Syn, CGA, and NCAM. This case was stage IIB (T2b, N0, M0), and the patient was alive at the time of the last examination 3 years after the initial diagnosis (**Fig. 3A-F**). The other case was LCNEC with adenocarcinoma. Staining was

Table 2. Positivity score of the tumor cell population and stain strength (grade) for insulinoma-associated protein 1 in cervical neuroendocrine neoplasms and non-neuroendocine neoplasms

	No.	No. of cases with tumor cell population positivity score for INSM1 (score)				Stain strength for INSM1 (grade)				Cases with tumor cell population positive for Syn/CGA/NCAM		
		0	1	2	3	0	1+	2+	3+	Syn	CGA	NCAM
NEN												
SCNEC	8	0	1	1	6	0	1	0	7	6	4	4
LCNEC	5	0	1	3	1	0	0	1	4	4	3	4
SCNEC+LCNEC	1	0	0	0	1	0	0	0	1	1	1	1
NET G2	2	0	0	0	2	0	0	0	2	2	2	1
Total	16	0	2	5	9	0	1	1	14	13	10	10
Positivity rate		INSM1, 15/16 (94%) (positive score, 1 to 3; stain grade, 2+/3+)								Syn, 13/16 (81.3%)	CGA, 10/16 (62.5%)	NCAM, 10/16 (62.5%)
Non-NEN												
Squamous cell carcinoma	61	60	1	0	0	60	0	0	1			
Adenocarcinoma	23	22	1	0	0	22	0	0	1			
Adenosquamous carcinoma	2	2	0	0	0	2	0	0	0			
CIN3	2	2	0	0	0	2	0	0	0			
Total	88	86	2	0	0	86	0	0	2			
Positivity rate		INSM1: 2/86 (2.3%) (positive score, 1 to 3; stain grade, 2+/3+)										

NEN, neuroendocrine neoplasia; INSM1, insulinoma-associated protein 1; Syn, synaptophysin; CGA, chromogranin A; NCAM, neural cell adhesion molecule; SCNEC, small cell neuroendocrine carcinoma; LCNEC, large cell neuroendocrine carcinoma; CIN3, cervical intraepithelial neoplasia, grade 3.

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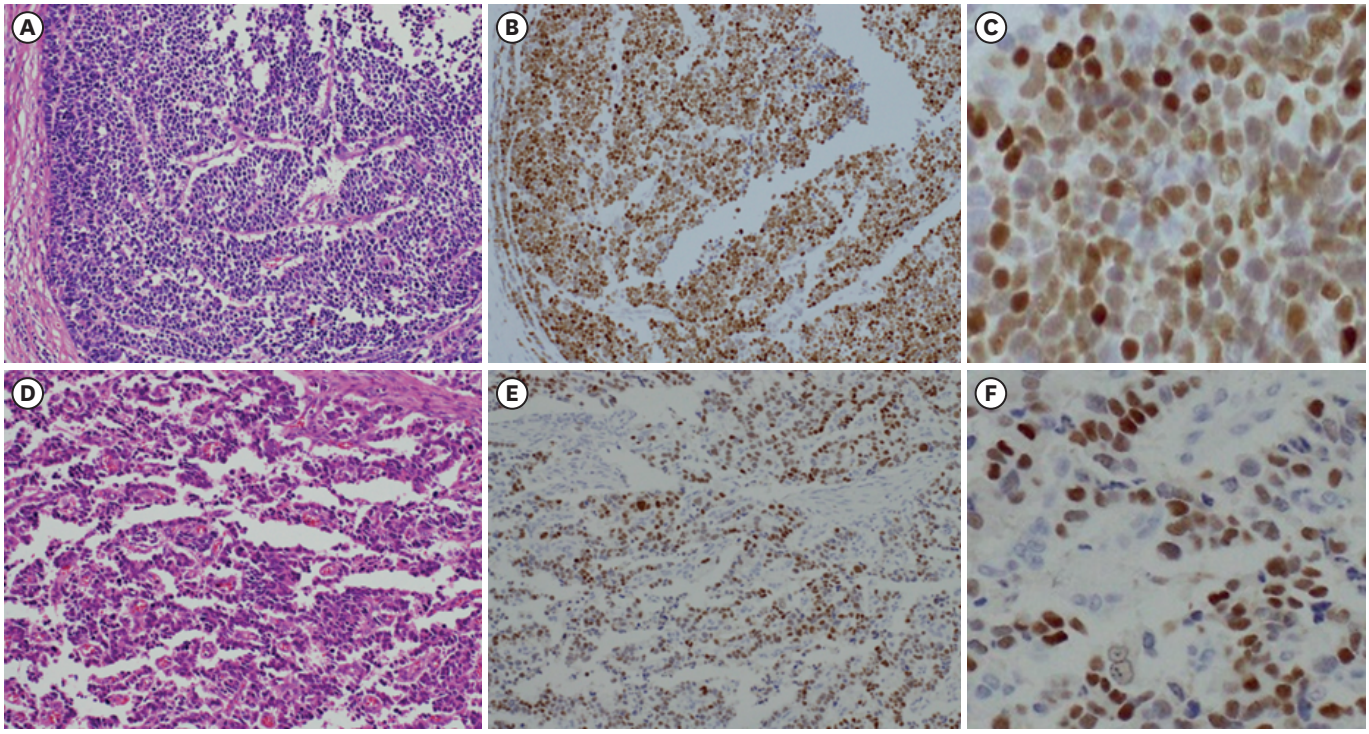

Fig. 2. INSM1 immunohistochemistry for cervical neuroendocrine neoplasia.
Small cell carcinoma of cervix: (A) H&E staining; (B, C) INSM1 immunohistochemical staining. Neuroendocrine tumor, grade 2 of cervix: (D) H&E staining; (E, F) INSM1 immunohistochemical staining.
INSM1, insulinoma-associated protein 1; H&E, hematoxylin and eosin.

negative for Syn and CGA, but partially positive for NCAM. This case was stage IIIC1 (T1b2, N1, M0). The patient recurred immediately after initial treatment, radical hysterectomy and adjuvant chemotherapy. The patient was alive with disease at the time of the last examination 2 years after the initial diagnosis (**Fig. 3G-L**).

Only one case with grade 1+ stain strength had small cell carcinoma, admix type. The tumor cell population positivity score was 2 for INSM 1. This case was diagnosed by being positive for Syn and morphological features.

Two of the 88 cases (2%) of cervical non-NEC were positive for INSM1 (**Fig. 4**). One case was adenocarcinoma with score 1 and grade 3+ for INSM1 (**Fig. 4A and B**). The positive cells with stain strength of grade 3+ were clearly located on the basal side of the glandular ducts in the tumor. Stainings for Syn, CGA, and NCAM were negative. This case was adenocarcinoma, HPV-associated (HPV type 16) and stage IB1 (T1b1, N0, M0). Follow-up data were not available after the initial diagnosis. The other case was squamous cell carcinoma with score 1 and grade 3+ for INSM1; stainings for Syn, CGA, and NCAM were negative (**Fig. 4C and D**). INSM1-positive cells with stain strength of grade 3+ were randomly scattered throughout the typical morphology of squamous cell carcinoma. This case was HPV-negative and stage IIA1 (T2a1, N0, M0); no follow-up data after the initial treatment were available.

Positive INSM1-based IHC for cervical cancer was defined as a tumor cell population positivity score of 1 to 3 and a stain strength grade of 2+ or 3+. According to this definition, the characteristics of INSM1 IHC for cervical cancer were as follows: sensitivity, 94%;

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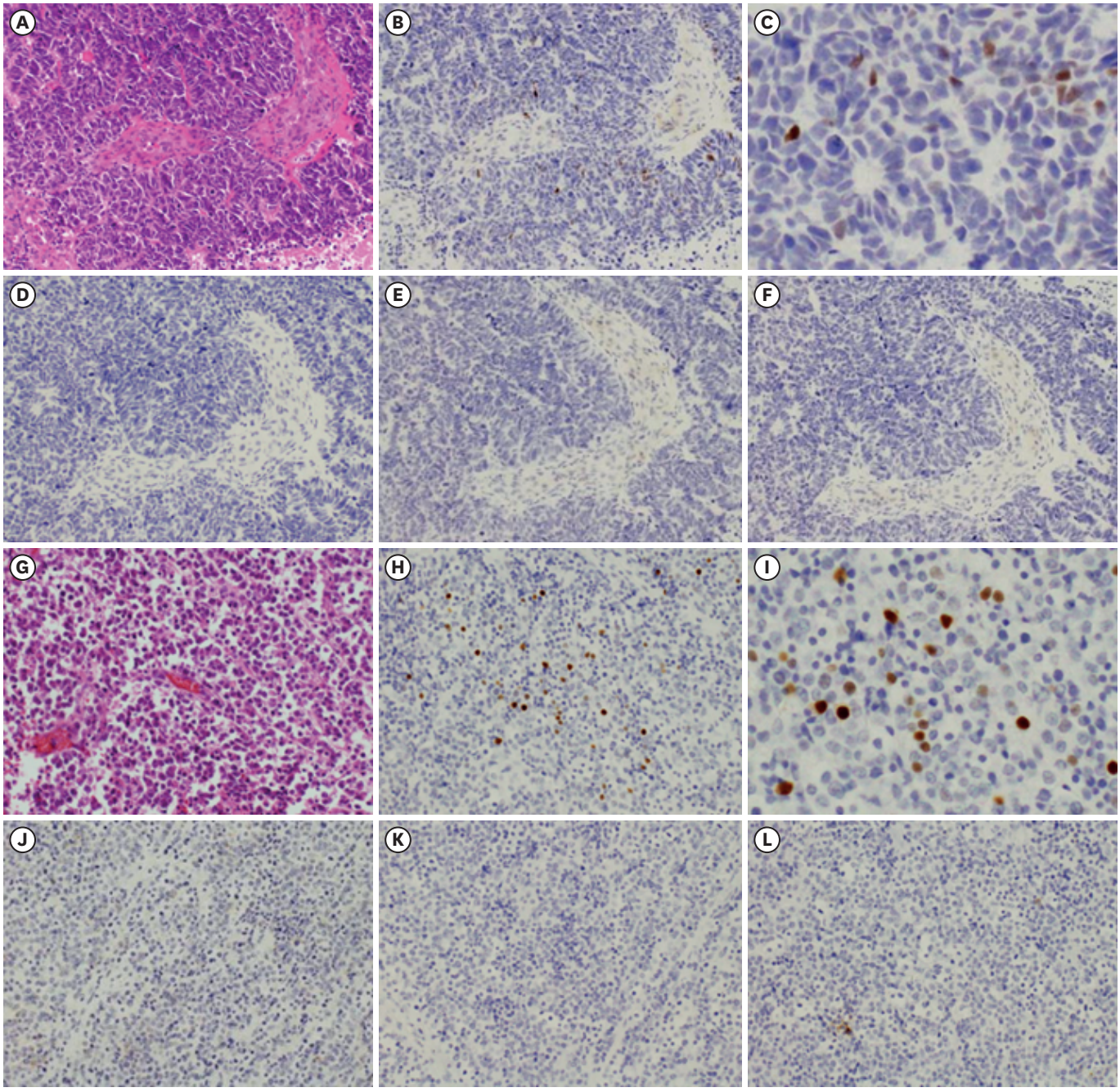

Fig. 3. Cervical neuroendocrine carcinoma with a tumor cell population positivity score 1.
Small cell carcinoma of the cervix: (A) H&E staining; (B, C) INSM1 immunohistochemical staining (stain strength, grade 3+); (D) Syn immunohistochemical staining; (E) CGA immunohistochemical staining; (F) NCAM immunohistochemical staining, LCNEC of the cervix (G) H&E staining, (H, I) INSM1 immunohistochemical staining (stain strength, grade 3+), (J) Syn immunohistochemical staining, (K) CGA immunohistochemical staining, (L) NCAM immunohistochemical staining.
H&E, hematoxylin and eosin; INSM1, insulinoma-associated protein 1; Syn, synaptophysin; CGA, chromogranin A; NCAM, neural cell adhesion molecule.

specificity, 98%, positive predictive value, 88%; and negative predictive value, 99%. The sensitivities of the other neuroendocrine markers for cervical NEN were as follows: Syn, 81%; CGA, 63%; and NCAM, 63%. The rate that was positive for 2 or 3 of Syn, CGA, or NCAM, which is a classic diagnostic method, was 63%.

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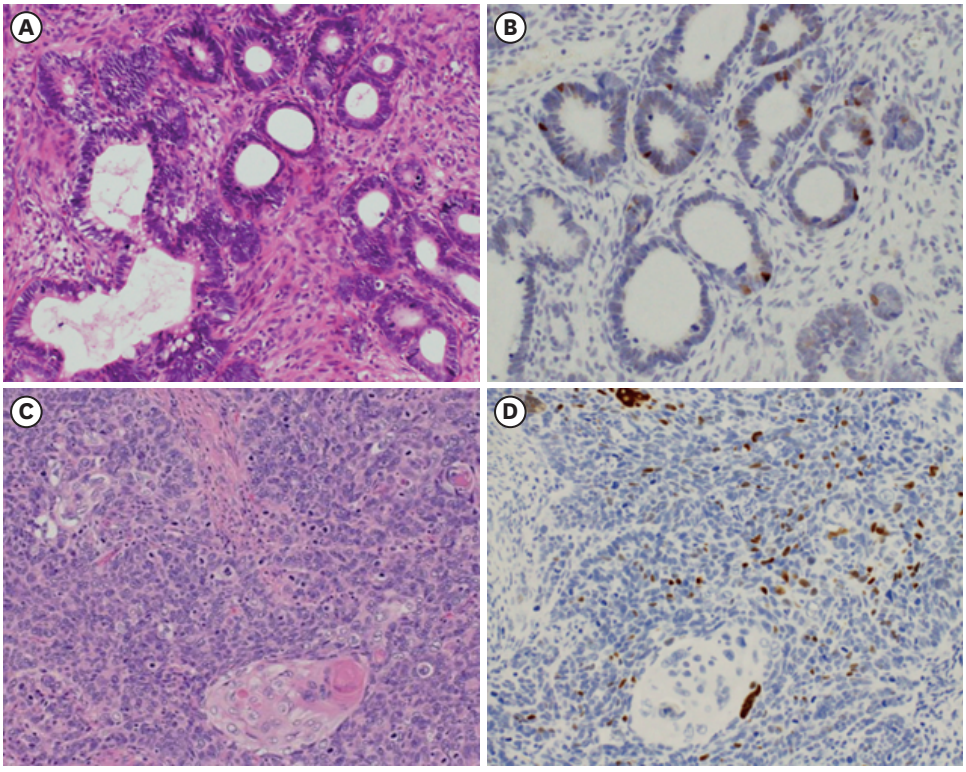

Fig. 4. Cervical non-neuroendocrine neoplasia with positive cells for INSM1.
Adenocarcinoma, usual type: (A) H&E staining and (B) INSM1 immunohistochemical staining. Squamous cell carcinoma: (C) H&E staining and (D) INSM1 immunohistochemical staining.
INSM1, insulinoma-associated protein 1; H&E, hematoxylin and eosin.

DISCUSSION

INSM1 is abundantly expressed in fetal developing neuronal and neuroendocrine tissue, but it is significantly reduced or restricted in adult tissues [14,15]. In normal adult tissues, expression has been confirmed in endocrine cells of tissues such as the adrenal medulla, pancreatic islets, gastrointestinal enterochromaffin cells, and cells thought to be endocrine cells in the normal bronchial epithelium of the lungs and non-neoplastic prostate glands [16]. Fujino et al. [15] reported that NE differentiation was balanced between differentiation-suppressing transcription factors, such as Hes1, and differentiation-promoting transcription factors, such as INSM1 and human achaete-scute homolog 1 (hASH1), as seen in the normal lung epithelial system. The Notch1-Hes1 signaling pathway suppressed NE differentiation through the inhibition of transcription factors, such as INSM1 and hASH1. Meanwhile, INSM1 promotes NE differentiation accompanying the enhanced expression of transcription factors, such as hASH1. Recently, several publications reported the diagnostic efficacy of INSM1 IHC as a neuroendocrine marker [10,15,16]. In the current study, RNA sequences in TCYIK showed high expression of INSM1. Similarly, the TPM level of hASH1, which is downstream of INSM1 in neuroendocrine differentiation, was highly expressed in TCYIK (TPM level, 217), whereas the TPM levels of SiHa and HeLa did not show any expression (TPM level, 0). As for Hes1, which suggests suppression of INSM1-hASH1, TPM of TCYIK was low (TPM level, 1.051), but it was high for SiHa and HeLa (TPM level, 44.38 and 14.18, respectively) (**Fig. S3**). INSM1 immunoreactivity was shown only in the TCYIK cell line, but

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not in SiHa and HeLa cell lines (**Fig. 1**). These results showed that only TCYIK cells expressed INSM1 protein. At the same time, the antibody's accuracy was assured.

Our previous report showed that sensitivity of INSM1 IHC was 95% in 37 cases of NEC of the uterine cervix [10]. In these cases, the sensitivities of Syn, CGA, and NCAM were 86%, 86%, and 68%, respectively. In a subsequent study, we evaluated the diagnostic efficacy of INSM1 for cervical NEC [10]. However, in that study, we performed IHC by a manual procedure in accordance with the approach used in our previous work, which made it difficult to evaluate stain strength [10]. In the present study, all processes without antigen retrieval were performed by an automated IHC system, which enabled us to compare the stain strengths and therefore evaluate the specificity accurately. In the present study, IHC was performed in 88 cases of non-NEN of the uterine cervix and showed sensitivity of 94% and specificity of 98%. These results demonstrate the high accuracy of INSM1 as a neuroendocrine marker for uterine cervical cancer. In the recent studies, the sensitivity and specificity of INSM1 for cervical NEC were similar, with sensitivity of 92% and specificity of 98% [17,18].

Previously reported data showed that positive rates for traditional NE markers, i.e., Syn, CGA and NCAM, of cervical NEC were 64%–96%, 32%–85%, and 68%–88%, respectively [10,19-21]. Regarding the accuracy of NEN diagnosis, among INSM1 alone, INSM1 combined with other neuroendocrine markers, and combinations of neuroendocrine markers other than INSM1, INSM1 alone showed the highest sensitivity for the diagnosis of cervical NEN in this study. INSM1 plus morphology seemed to be sufficient for the diagnosis of cervical NEN.

In the present study, only one case, which was SCNEC admixed with squamous cell carcinoma in which a biopsy specimen was obtained, showed INSM1 stain strength of grade 1+. This case was diagnosed by being positive for Syn (and negative for GCA and NCAM) and by its morphological features. Although staining for INSM1 was confirmed in this case, in clinical practice it would be difficult to make a definitive diagnosis with this weak staining (grade 1+). In 3 biopsy cases in the current study, one was the above case, and another case showed INSM1 stain strength of grade 2+. In 13 cases that underwent hysterectomy or conization, 2 cases showed INSM1 stain strength of grade 2+, one was hysterectomy and another case was conization. We consider that formalin fixation and dealing with the specimens immediately after resection or biopsy may affect INSM1 stain strength.

Recently, in small cell lung cancer, it was suggested that there were INSM1-low and INSM1-high subgroups. INSM1-low suggests low chemosensitivity or a poor prognosis [22]. The current data did not show a relationship between INSM1 expression status and prognosis (**Table S2**). However, in 2 cases of stage IVB, one case showed grade 1+ and score 2 INSM1 staining, and another case showed grade 2+ and score 2. It may be noteworthy that, in these IVB cases, staining of both was weak. A large number of cases would be worth investigating.

In the present study, there were 2 cases of INSM1-positive cervical non-NEN. One case was adenocarcinoma, HPV-associated; this tumor contained a small number of cells with stain strength grade 3+. Therefore, it may have been an ordinary type adenocarcinoma with a component that showed intestinal type or intestinal-type differentiation; the positive cells were located on the basal side of the glandular ducts in the tumor, suggesting the possibility of symbiotic neuroendocrine cells. However, these cells were negative for Syn, CGA, and NCAM. Another INSM1-positive case was SCC, HPV-independent. This tumor showed the typical characteristics of SCC on H&E staining and was negative for Syn, CGA, and NCAM.

Morphologically, the tumor was a typical squamous cell carcinoma; however, INSM1-positive cells with stain strength of grade 3+ were randomly scattered throughout the tumor, suggesting that some cells may have differentiated into neuroendocrine cells. Some tumors might contain INSM1-positive cells in highly differentiated areas, as in the first case. In some cases, neuroendocrine differentiated cells might appear suddenly in the tumor, as in the second case.

Very recently, it was reported that one-third of lung squamous cell carcinomas with basaloid features were positive for INSM1 [23]. Thus, INSM1 may be a pitfall in the diagnosis of certain subtypes of cervical cancer.

IHC for INSM1 was tried in endometrial cancer (4 cases of endometrial NEN and 59 cases of endometrial non-NEN). In 4 cases of NEN, it was difficult to diagnose and classify them as small cell carcinoma or LCNEC according to WHO (2020), although all cases were positive for Syn, CGA, and NCAM. Furthermore, INSM1 staining was not clearly comparable with that of cervical NEN, as follows: tumor cell population positivity score 1 in 3 cases and negative in 1 case; stain strength grade 1 in 3 cases and negative in 1 case. One case that was INSM1-negative was also suspected to have undifferentiated carcinoma.

In addition, in endometrial non-NEN, 10 of 59 cases (17%) in the present study were positive for INSM1 when the same definition of positivity as for cervical NEN was used. Four of 37 cases (11%) of endometrioid adenocarcinoma and 6 of 15 (40%) cases of carcinosarcoma were positive for INSM1 (**Tables S3** and **S4**). The results suggest that there are many false-positive cases of INSM1 in non-endometrial NEN. All authors discussed about these problems and concluded that endometrial carcinoma should be excluded from this study in this time.

The INSM1 staining strength in a small number of cases of endometrial NEC was often reported to be weak and less sensitive [17]. Zou et al. [18] reported staining by neuroendocrine markers of 138 cases of endometrial non-NEN, and tumor was positive in 6 cases (4%) for INSM1, 44 cases (32%) for CGA, 25 cases (18%) for Syn, and 23 cases (17%) for NCAM. In addition, 4 of 10 cases (40%) of undifferentiated endometrial carcinoma were positive for INSM1 [18]. In another study, Moritz et al. [24] reported that 17 of 26 cases (65.4%) of endometrioid adenocarcinoma and 10 of 10 cases (100.0%) of carcinosarcoma were stained by one or more of the neuroendocrine markers Syn, CGA, and NCAM. These results suggest that INSM1 is not as accurate as the other neuroendocrine markers for diagnosing endometrial NEN.

In this study, the specificity of INSM1 for cervical cancer was clearly demonstrated by using an automated immunostaining system. INSM1 showed very high specificity for cervical NEN, suggesting that INSM1 is very useful for the diagnosis and differential diagnosis of NEN. However, the limitation is that the non-NENs included in this study were those diagnosed as adenocarcinoma or squamous cell carcinoma, and grade and subtypes were not considered. Since staining for these subtypes may be a pitfall, the next study is to examine cases that show such histological features.

In conclusion, INSM1 IHC is an adjunctive diagnostic method with excellent specificity and sensitivity in the diagnosis of cervical NEN. The present study showed that higher specificity can be obtained if morphological evaluation is also performed.

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SUPPLEMENTARY MATERIALS

Table S1
Definition of positivity of the tumor cell population and stain strength

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Table S2
Patient' clinical and histological information and immunohistochemical judgement

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Table S3
Types of tumors identified in patients diagnosed endometrial non-neuroendocrine carcinoma (n=59)

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Table S4
The score of positivity of the tumor cell population (score) and stain strength (grade) for INSM1 in endometrial carcinoma

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Fig. S1
Image of the strength (grade) of insulinoma-associated protein 1 staining.

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Fig. S2
PCA scatter plot and heatmap of TCYIK, SiHa, and HeLa.

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Fig. S3
Transcripts per million level of INSM1, hASH1, and Hes1 on RNA sequence.

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