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Immunohistochemical, Biochemical and Immunoelectron Microscopic Analysis of Antigenic Proteins on Neuroendocrine Cell Tumors Using Monoclonal Antibody HISL-19

Kazuo Shimizu¹, Shigeki Namimatsu², Wataru Kitagawa¹, Haruki Akasu¹,
Keisuke Takatsu¹, Yuichi Sugisaki² and Shigeo Tanaka¹

¹Department of Surgery II, Nippon Medical School

²Department of Surgical Pathology, Nippon Medical School

Abstract

The monoclonal antibody HISL-19 was originally generated after immunizing BALB/c mice with human islet cells. We used this antibody to study a wide variety of neuroendocrine (NE) and non-NE tumors by immunohistochemical, immunoelectron microscopic, and biochemical (Western blotting) techniques. Of the thyroid tumors, HISL-19 specifically immunoreacted with medullary carcinoma of the thyroid (MCT); of the pancreatic tumors, it reacted with islet cell tumors such as insulinomas and a gastrinoma; of the adrenal tumors, it reacted with pheochromocytoma. HISL-19 showed particularly strong immunoreactivity to a gross granular material at the perinuclear area in the MCT and malignant pheochromocytoma but not in the benign pheochromocytoma, although the latter cells showed a faint and homogenous positive reactivity in the cytoplasm. The strongly HISL-19-positive material was found to consist of newly synthesized antigenic proteins with a molecular weight between 60 and 65 kilodaltons (kDa) by Western blotting. Immunoelectron microscopic analysis revealed that this antigenic protein was located in the secretory granules that appear markedly in malignant endocrine tumors, usually located close to the nucleus. Thus, HISL-19 is a useful and specific marker for the immunohistochemical diagnosis of NE cell tumors. The specific antigenic proteins of HISL-19 were defined in MCT and malignant pheochromocytoma. These proteins are speculated to be actively synthesized and more highly produced in the secretory granules of malignant endocrine tumors than benign ones. Thus, a preoperative immunohistochemical study using HISL-19 might be useful for predicting the grade of malignancy of endocrine malignant tumors and thus help determine an appropriate operative procedure, in addition to being a useful marker of neuroendocrine cell tumors. (J Nippon Med Sch 2002; 69: 365–372)

Key words: monoclonal antibody HISL-19, neuroendocrine tumor, immunohistochemistry, immunoelectron microscopic study, tumor marker

Introduction

Neuroendocrine (NE) tumors originating from the neural crest have common embryonic pathways and possess unique properties. They produce excessive peptide hormones, cause typical clinical symptoms, and are sometimes found in familial genetic syndromes, such as multiple endocrine neoplasia (MEN) type I or II a, b.

We have reported immunohistochemical studies of NE tumors from various endocrine organs using monoclonal antibodies¹⁻¹⁴ for clinical applications. Among those monoclonal antibodies, A 2 B 5¹⁵ was found to react with the GQ ganglioside, which is expressed on APUD cells and their tumors, as its common antigenic determinant^{12,15,16}. We have reported the characteristics of this murine monoclonal IgM antibody not only in terms of its immunohistochemical and biochemical properties, but also its clinical usefulness.

In this paper, we used another monoclonal antibody, HISL-19¹⁷, which was originally generated by Srikanta et al¹⁷ by immunizing BALB/c mice with a human islet cell preparation and was similar to A 2 B 5 in that it is a marker of NE cell tumors. This antibody was reported to be a cellular IgG protein. So far, the antigen for HISL-19 has been reported to be a protein with a molecular weight of 120, 69, 67, and 56 kilodaltons (kDa)¹⁷ located in the intracellular space of islet cells. Using light and electron microscopic immunohistochemical studies and biochemical analysis, we found novel antigenic determinants of HISL-19 associated with endocrine tumors. Furthermore, we discuss how this antibody could be clinically useful in the field of endocrine surgery.

Materials and methods

For the light microscopic immunohistochemical (LIH) studies, formalin-fixed, paraffin-embedded surgical specimens from various endocrine and non-endocrine tumors were sliced into to 4- μ m-thick sections. Immunohistochemical (IH) staining was performed using the indirect immunofluorescence or Strepto-Avidin Biotin peroxidase (SAB-PO) complex

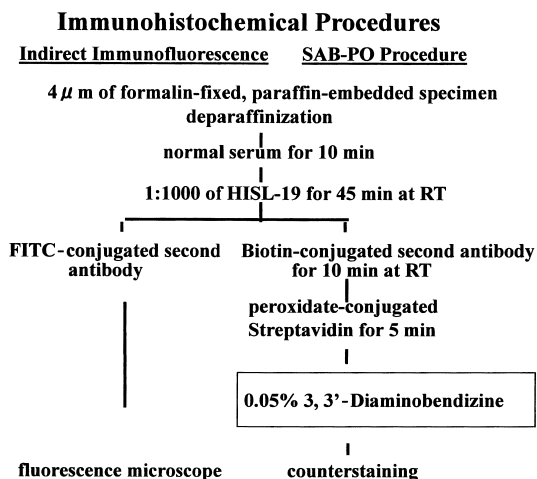


Fig. 1 The immunohistochemical procedure using indirect immunofluorescence or SAB-PO. The specimens were prepared from formalin-fixed, paraffin-embedded 4- μ m sections. The HISL-19 was diluted 1:1000 with PBS containing 10% of fetal calf serum. The peroxidase gave rise to a color reaction when diaminobenzidine was used as a substrate in the SAB-PO Procedure.

method. The immunohistochemical procedures are shown in **Fig. 1**.

For the immunoelectron microscopic (IEM) study, a formalin-fixed, paraffin-embedded specimen from a medullary carcinoma of the thyroid (MCT) that had already been confirmed to show a positive reaction to HISL-19 in an LIH study was used to localize the HISL-19-positive staining to cytoplasmic organelle. The immunoelectron microscopic specimens were prepared using the pre-embedded procedure.

To analyze the molecular weight of the antigenic protein of HISL-19, Western blotting was carried out using samples prepared from papillary thyroid carcinoma (PTC), MCT, Cushing's syndrome, and benign pheochromocytoma. **Fig. 2** shows how the protein extracts were prepared from these tissues. After subjecting the homogenized fresh surgical specimens to three centrifugation steps, the final supernatant, representing the cytosomal fraction, was used for analysis.

Preparation of Protein Extract from Tissue

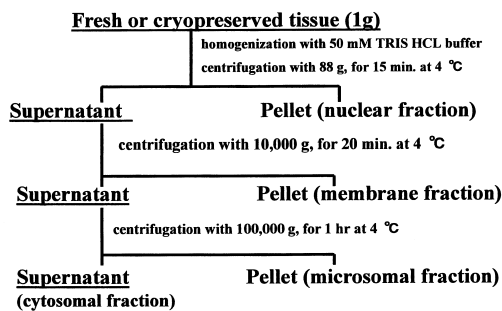


Fig. 2 Preparation of protein extract from tissues for the Western blotting. Fresh or cryopreserved tissue directly obtained at surgery was homogenized in 50 mM TRIS-HCl buffer. After three subsequent centrifugation steps, the final supernatant was considered to be the cytosomal fraction and was processed for biochemical analysis of the target protein's molecular weight.

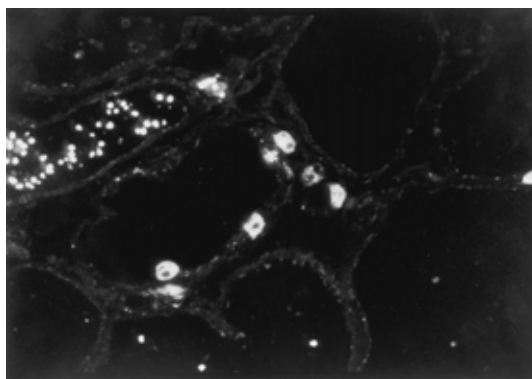


Fig. 3 Immunoreactivity of HISL-19 to normal thyroid by the indirect immunofluorescence procedure. Only the C cells reacted with HISL-19, while the follicular epithelial cells did not.

Results

1. LIH study for NE and non-NE cells and their tumors

1) Thyroid: In the normal thyroid gland, HISL-19 reacted only with C cells (Fig. 3). Of the thyroid tumors, HISL-19 reacted strongly with 8 of 9 cases of MCT, which is known to originate from C cells (Fig. 4). Of 11 cases of papillary carcinoma, 13 of follicular adenoma, and 5 of Hashimoto's thyroiditis, all were negative for HISL-19 except for one weakly positive case for each disease. All follicular carcinomas,

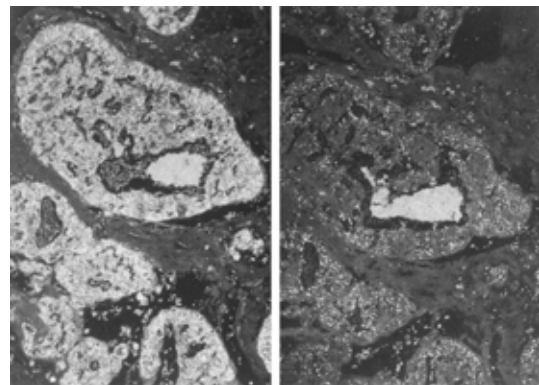


Fig. 4 Immunoreactivity of HISL-19 to medullary carcinoma of the thyroid. The picture on the left shows a positive reaction to HISL-19. The picture on the right is an identical section immunostained with another monoclonal antibody as a negative control. Both sections were stained by the indirect immunofluorescence procedure.

anaplastic carcinomas, and thyroid malignant lymphomas tested were completely negative for HISL-19 (Table 1).

2) Adrenal gland: Using the SAB-PO method, HISL-19 bound only to the adrenal medulla and not to the adrenal cortex in the normal adrenal gland (Fig. 5 b). Of the adrenal tumors, it bound to pheochromocytomas, which originate from the adrenal medulla (Fig. 5 a), but not to 5 cases of primary aldosteronism, one case of Cushing's syndrome, and 5 cases of non-functioning cortical adenoma, which are all derived from the adrenal cortex. In the pheochromocytomas, the immunoreactivity of the malignant tumors was obviously stronger than that of the benign ones (Table 1). The staining was weakly positive in the whole cytoplasm in the benign tumors while the malignant tumors showed strongly positive staining in a gross granular pattern, in an area close to the nucleus and/or in the whole cytoplasm (Fig. 5 a).

3) Pancreas: The immunoreactivity of HISL-19 to the normal pancreas using the immunofluorescence method showed clearly positive staining only of the islet cells but not of the acinar cells that surround the islet (Fig. 6). Of the pancreatic tumors, HISL-19 bound to insulinoma (Fig. 7) and malignant gastrinoma (Fig. 8), but not to acinar cell carcinoma (Table 1).

Table 1 Immunoreactivity of HISL-19 to the thyroid, adrenal, and pancreatic diseases

Specimens (cases)	Reactivity			
	2 +	1 +	±	-
<u>Thyroid</u>				
Papillary carcinoma (11)		1	2	8
Follicular carcinoma (6)			1	5
Medullary carcinoma (9)	8	1		
Anaplastic carcinoma (6)			2	4
Follicular adenoma (13)		1	3	9
Hashimoto's thyroiditis (5)		1	1	3
Malignant lymphoma (3)				3
<u>Adrenal</u>				
Primary aldosteronism (5)				5
Cushing's syndrome (5)				5
Non-functioning cortical adenoma (1)				1
Pheochromocytoma				
Benign (3)		3		
Malignant (2)	2			
<u>Pancreas</u>				
Insulinoma (2)	2			
Gastrinoma (1)	1			
Acinar cell carcinoma (5)			1	4

Immunoreactivity of HISL-19 to thyroid, adrenal, and pancreatic diseases. Medullary carcinoma of the thyroid of the thyroid tumors, pheochromocytoma of the adrenal tumors, and islet cell tumor of the pancreatic tumors were specifically positive to HISL-19, with the exception of a few cases of the thyroid tumors, which are considered to be a non-specific reaction. Malignant pheochromocytoma showed a stronger reaction with HISL-19 than did benign pheochromocytoma.

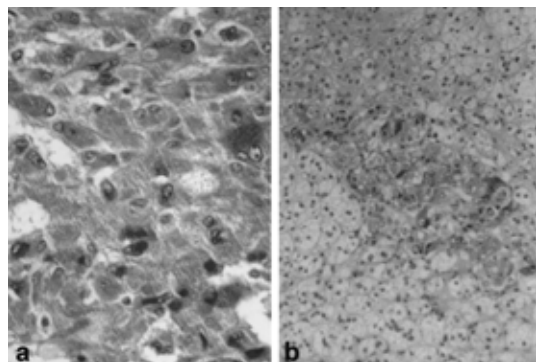


Fig. 5 Immunoreactivity of HISL-19 to a malignant pheochromocytoma (a) and normal adrenal tissue (b) by the SAB-PO procedure. The immunoreactivity in the malignant pheochromocytoma (a) showed different patterns among the tumor cells. Some cells showed a strong reaction throughout the cytoplasm while others showed a strong positive cluster near the nucleus. All the positive tissues showed the staining of gross granular material. The picture on the right shows positive immunoreactivity at the adrenal medulla but not in the cortical area surrounding the medullary area.

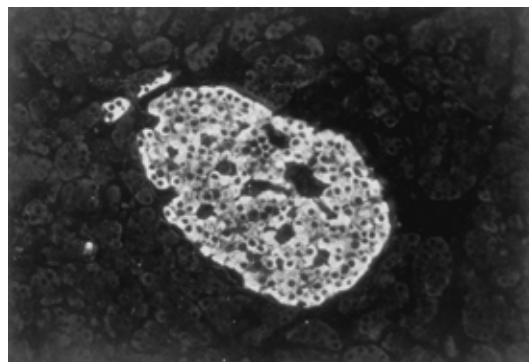


Fig. 6 Immunoreactivity of HISL-19 to normal pancreas stained by the indirect immunofluorescence method. The HISL-19 only binds to pancreatic islets, but not to normal pancreatic acinar cells surrounding the islet.

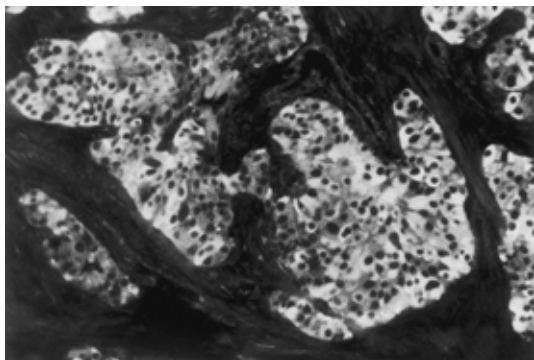


Fig. 7 Immunoreactivity of HISL-19 to insulinoma, which proliferates in the fibrous connective tissue. The tumor nests are clearly positive to HISL-19 by the indirect immunofluorescence procedure.

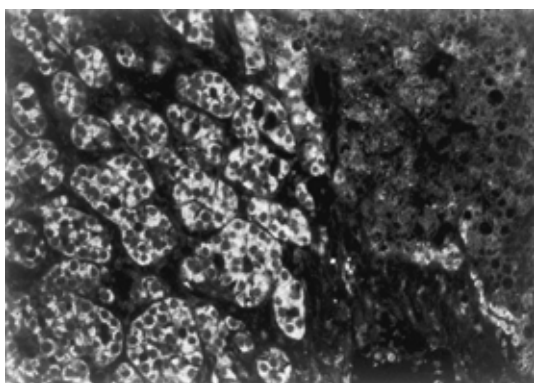


Fig. 8 Immunoreactivity of HISL-19 to metastatic gastrinoma in the liver. The HISL-19-positive malignant gastrinoma is shown on the left side of this picture while normal liver cells, which are negative to HISL-19, are shown on the right side. The positive staining in the cytoplasm of the malignant tumor cells shows a different pattern from that of the normal islet and insulinoma, which are benign cells.

2. IEM study for MCT

A MCT that was strongly positive for HISL-19 in the LIH study was further analyzed by IEM study (Fig. 9). Samples prepared using the pre-embedding method were clear enough for recognition of the cytoplasmic organelles such as the nucleus, nucleoli, rough endoplasmic reticulum, secretory granules, and lysosomes by routine uranyl acetate and lead citrate double staining (Fig. 9a). The HISL-19-positive gross granular material located at the perinuclear

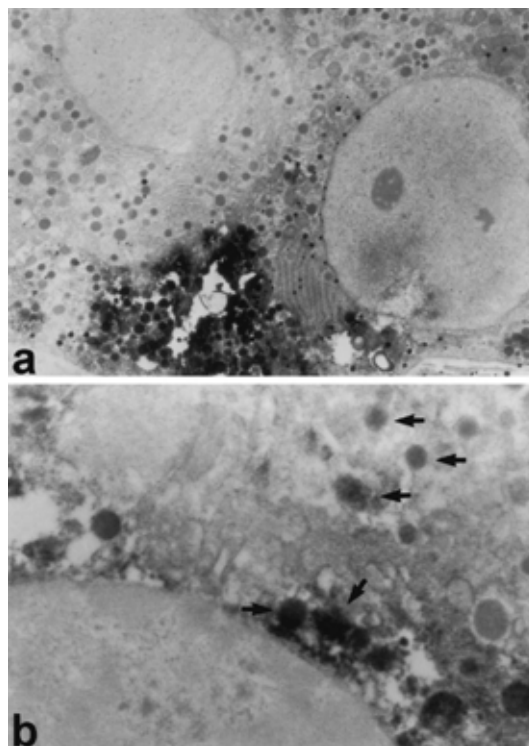


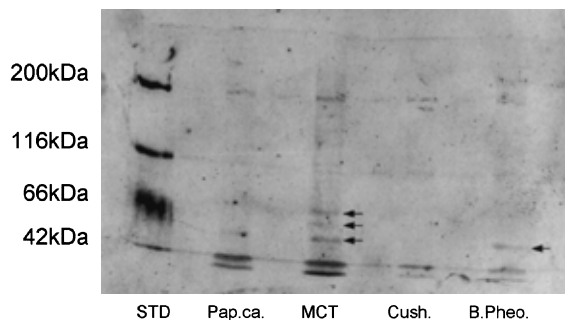
Fig. 9 Immunoelectron microscopic findings of HISL-19 on MCT. Panel (a) shows uranyl acetate and lead citrate double staining of MCT. The cytoplasmic organelles such as the nucleus, nucleoli, lysosomes, endoplasmic reticulum, and Golgi apparatus can be recognized (2,000 \times). Panel (b) shows the IEM results of HISL-19 reactivity on MCT in a highly magnified view without counterstaining (60,000 \times). HISL-19-positive materials (arrows), which is considered to be secretory granules, are seen at the perinuclear zone.

area in the LIH study was localized to the secretory granules without the routine staining (Fig. 9 b). These positive granules showed a tendency to decrease in number as they were transferred to the apical surface of the C cell.

3. Evaluation of the molecular weight of the HISL-19 antigen using Western blotting

The specific antigenic determinants of HISL-19 in endocrine cell tumors originating from the neural crest were detected using Western blotting. Three clear bands were found with molecular weights between 55 and 65 kDa in MCT, but not in PTC or Cushing's syndrome, which were immunohistochemically negative for HISL-19. The upper two bands, whose

Western Blotting using monoclonal antibody HISL-19



STD :Standard Marker
Pap.ca. :Papillary Carcinoma of the Thyroid
MCT :Medullary Carcinoma of the Thyroid
Cush. :Cushing's Syndrome (Cortical Adenoma)
B.Pheo. :Benign Pheochromocytoma

Fig. 10 Biochemical analysis by Western blotting for investigating the molecular weight of the HISL-19 antigen on various tumors, including HISL-19-positive tumors (MCT: strongly positive, B. Pheo: weakly positive). Of three bands, the upper two, with a molecular weight between 60 and 65 kDa, were seen in MCT but not in other tumors, while the one band found in benign pheochromocytoma corresponded with the lowest band seen in MCT, and had an estimated molecular weight of around 55 kDa.

molecular weights fell between 60 to 65 kDa, were newly synthesized, and were only found in MCT, and not in benign pheochromocytoma, which was weakly positive for HISL-19 and showed just one reactive band with a molecular weight of 55 kDa (**Fig. 10**).

Discussion

Compared with polyclonal antibodies, monoclonal antibodies, whose isolation was developed by Köhler & Milstein¹⁸, proved to have better specificity, homogeneity, reproducibility, and reliability. Therefore, the availability of monoclonal antibodies both advanced the field of fundamental immunology and provided diagnostic and therapeutic applications in clinical settings. In particular, immunohistochemical studies for the clinical application of monoclonal antibodies as tumor markers have played an important role in

determining tumor origins and classifying tumors biochemically.

We have previously reported the usefulness of various monoclonal antibodies as markers for benign and malignant endocrine tumors^{1,2,4,7} including thyroid tumors^{9,12}. Of the thyroid tumors, the monoclonal antibody PKK-1⁹ reacts with well-differentiated papillary carcinoma of the thyroid, while 4F2¹² reacts with anaplastic carcinoma of the thyroid. Our studies also determined A 2 B 5's common antigen to be the GQ ganglioside, which is expressed on the cell membrane of neuroendocrine tumors¹³. Although both A 2 B 5 and the human islet cell monoclonal antibody, HISL-19, are markers of NE tumor cells, A 2 B 5's antigenic determinant is destroyed by formalin fixation, whereas HISL-19 can react with formalin-fixed, paraffin embedded surgical specimens and thus may offer a definite advantage. Therefore, this antibody will permit retrospective immunohistochemical studies to be performed post-operatively on formalin-fixed specimens.

HISL-19 was first established and reported by Srikanta et al¹⁷, and its potential usefulness for neuroendocrine cell tumors was subsequently studied by Krisch and coworkers¹⁹ and Neuhold and his colleagues²⁰. The HISL-19-positive endocrine tumors found in our study were almost the same as those reported by these authors^{19,20}.

In further studies of HISL-19 with endocrine tumors, Deftos et al²¹ studied the immunohistochemical reactivity of the HISL-19 antigen and chromogranin A (CgA) in 14 cases of MCT, and compared them with the well-known MCT marker, anti-calcitonin antibody. They found all three to be commonly expressed. Krisch and his colleagues²² described immunohistochemical and biochemical differences between the HISL-19 antigen and Cg proteins A, B, and C using samples prepared from neuronal and peptide-hormone-producing cells and their tumors. In their study, although HISL-19 antigen was expressed in Cg-protein-producing cells, it differed from Cg proteins in its distribution in the cytoplasm, molecular weight, and lack of cross reactivity with Cg-specific antibodies.

Our series of LIH, IEM, and biochemical studies has given rise to some more different findings and newer

information than that reported by groups at other institutions. First, we found different staining patterns between benign and malignant pheochromocytomas. Specifically, faint homogenous HISL-19-positive staining was found in the whole cytoplasm in the benign pheochromocytoma, while strongly positive staining of a gross granular material was found at the perinuclear area in the malignant pheochromocytomas. Similar immunoreactive patterns were described by Bordi et al²³ and Neuhold et al²⁴. In their papers, this HISL-19-positive gross granular material seen at the perinuclear area was described as "cluster-type or focal immunoreactive aggregates"²³ or "pure cluster type"²⁴ and was observed more in less-differentiated tumors²³ and malignant C cells²⁴. Using a monoclonal antibody against chromogranin, Varndell et al²⁵ found antigenic proteins in a wide variety of endocrine cells and their tumors, such as a pheochromocytoma, gastrinoma, MCT, and insulinoma, and localized them electron microscopically to endocrine secretory granules.

Second, our IEM study provides the first report of the precise localization of the HISL-19-positive protein to the secretory granules and their concentration at the perinuclear area. As they migrate to the apical surface of the tumor cells, these positive granules tended to gradually decrease in number. These phenomena suggest that malignant pheochromocytomas actively synthesize and produce new proteins that are not produced in benign ones.

Third, the molecular weights of the new antigenic proteins appearing in malignancy were evaluated by Western blotting using a sample prepared from an MCT that showed a strongly positive HISL-19 immunoreactivity, similar to that of the malignant pheochromocytoma, and compared it with samples prepared from HISL-19 fairly positive. As shown in **Fig. 10**, two upper bands with molecular weights between 60 and 65 kDa were clearly seen in the MCT, but not in the adrenocortical tumor, papillary carcinoma of the thyroid, or benign pheochromocytoma. This finding leads to the speculation that malignant tumors from endocrine organs actively synthesize and produce antigens, which we identified here as the cytoplasmic proteins of novel molecular weights.

Moreover, the above-mentioned findings can

be used to indicate the grade of the biological malignancy, where a strongly positive HISL-19 response would be associated with rapid tumor growth, high frequency of mitosis, and metastasis. Indeed, HISL-19 strongly positive MCT was associated with worse morbidity than was weakly positive MCT (manuscript in preparation).

If HISL-19 enabled a preoperative immunohistochemical diagnosis including aspiration biopsy cytology to be made for endocrine malignancy, a prediction of prognosis might be made. Accordingly, immunohistochemical findings could indicate the extent of tumor resection and the area of lymph node clearance and therefore guide the selection of an appropriate operative procedure. Further studies including analysis of the HISL-19 antigen mRNA expression are expected to clarify the usefulness of this remarkable protein as a specific marker for NE tumors. These studies are presently underway.

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