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Regular Article

Analysis of Insulinoma-Associated Protein 1 Expression in Pituitary Neuroendocrine Tumors

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Insulinoma-associated protein 1 (INSM1) is a representative diagnostic marker of neuroendocrine neoplasms (NENs); however, it has not yet been used to diagnose pituitary neuroendocrine tumors (PitNETs), according to the 2022 World Health Organization (WHO) classification of pituitary tumors. This study aimed to examine the expression of INSM1 using immunohistochemistry, in the various cell lineages of PitNET classified by hormone secretion and transcription factor expression. INSM1 expression in PitNETs (different subtypes) and normal pituitary tissues was immunohistochemically assessed. The results were interpreted as scores of 0 (negative), 1 (focally positive), or 2 (frankly positive), depending on the proportion of cell staining. Twenty-eight of 35 PitNET cases (80%) showed INSM1 positivity in their nuclei. The staining in each histological subtype of PitNETs was as follows: somatotroph tumors, score 0 = 3/5, score 1 = 1/5, score 2 = 1/5; lactotroph tumors, score 0 = 2/5, score 1 = 1/5, score 2 = 2/5; thyrotroph tumors, score 2 = 5/5; corticotroph tumors: score 1 = 1/9, score 2 = 8/9; gonadotroph tumors, score 0 = 2/10, score 1 = 0/10, score 2 = 8/10; and unclassifiable tumor, score 1 = 1/1. INSM1 expression in most PitNETs was obtained, similar to that in the normal pituitary gland; thus, INSM1 may maintain the characteristics of anterior pituitary cells and pituitary tumors.

Key words: carcinogenesis, neuroendocrine tumors, pituitary neoplasms, transcription factors, insulinoma-associated protein 1 (INSM1)

I. Introduction

Insulinoma-associated protein 1 (INSM1) is a transcription factor that induces differentiation in neuroendocrine cells or tumor cells in various organs, such as the pancreas, gastrointestinal tract, lungs, and pituitary gland, as well as in the nervous system [4, 6, 9]. Historically, pituitary neuroendocrine tumors (PitNETs) were classified based on hormone secretion; however, the 2022 World Health Organization (WHO) classification categorized tumors based on cell lineage, transcription factors, and other biomarkers [1]. INSM1 is a representative diagnostic marker of neuroendocrine neoplasms. However, it has not yet been used to categorize PitNETs, although its usefulness for diagnosis has been reported for various organs. INSM1 is highly expressed in medulloblastomas [8] and *INSM1* gene is included in the interaction network in tumorigenesis of malignant pheochromocytomas [11].

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Although INSM1 is expressed in normal pituitary cells [13], there are only a few sporadic reports on INSM1 expression in PitNETs [2, 3, 4, 9, 10]. In this study, we described the immunoexpression of INSM1 in PitNETs with consideration of correlation with cell differentiation, hormone secretion and transcription factor expression.

II. Materials and Methods

Thirty-five formalin-fixed paraffin-embedded surgical specimens obtained from Nippon Medical School between 2012 and 2018 were used in this study.

Paraffin sections were used for hematoxylin-eosin and immunohistochemical staining. The diagnosis of PitNETs was confirmed by two independent pathologists (C.I. and R.Y.O.). Sections were deparaffinized using xylene and graded concentrations of ethanol. Endogenous peroxidase was inactivated using 0.3% H_2O_2 in distilled water (DW). The sections were immunostained using an indirect peroxidase method with an antibody against each hormone, including the human growth hormone, prolactin, adrenocorticotropic hormone, thyroid-stimulating hormone, luteinizing hormone, follicle-stimulating hormone, and α-subunit, to confirm the subtypes and endocrinological activities of the PitNETs. Transcription factors, such as steroidogenic factor-1 (SF1), pituitary-restricted transcription factor

Table 1. *Antibodies used in this study*

Antibody Name	Nature	Host	Dilution	Antigen Retrieval	Vendor	Secondary Antibody Name	Dilution	Vendor	Visualization kit	Vendor
ACTH	Monoclonal	Mouse	1:400	—	DAKO	EnVision/HRP	undiluted	DAKO		
TSH	Monoclonal	Mouse	1:200	—	DAKO	EnVision/HRP	undiluted	DAKO		
FSH	Monoclonal	Mouse	1:50	PIER proteinase K	DAKO	EnVision/HRP	undiluted	DAKO		
GH	Polyclonal	Rabbit	1:400	—	DAKO	ECL anti-rabbit IgG	1:100	GE Healthcare	EnVision/HRP	DAKO
PRL	Polyclonal	Rabbit	1:400	—	DAKO	ECL anti-rabbit IgG	1:100	GE Healthcare	EnVision/HRP	DAKO
LH	Monoclonal	Mouse	1:200	—	IMMUNOTECH/ MBL	ECL anti-mouse IgG	1:100	GE Healthcare	EnVision/HRP	DAKO
a-subunit	Polyclonal	Rabbit	1:200	—	NIDDK, NIH	ECL anti-rabbit IgG	1:100	GE Healthcare	EnVision/HRP	DAKO
Tpit	—	Rabbit	1:300	HIER (autoclave)	Professor Jacques	Donkey Anti-Rabbit	1:300	Jackson	VECTASTAIN	Vector
				Target Retrieval Solution, Citrate pH 6	Drouin	IgG (H&L)-Biotin		Im-munoRe-search	Elite ABC kit	Laboratories
SF-1	Monoclonal	Mouse	1:100	HIER (autoclave)	Invitrogen	Goat anti-Mouse IgG	1:500	Fitzgerald	VECTASTAIN	Vector
				Target Retrieval Solution, Citrate pH 6		(H&L)-Biotin			Elite ABC kit	Laboratories
Pit-1	Monoclonal	Mouse	1:100	HIER (water bath)	Santa Cruz	Goat anti-Mouse IgG	1:100	Fitzgerald	VECTASTAIN	Vector
				Target Retrieval Solution, Citrate pH 6		(H&L)-Biotin			Elite ABC kit	Laboratories
CAM5.2	Monoclonal	Mouse	1:2	HIER (water bath) Target Retrieval Solution, Citrate pH 6	BD	EnVision/HRP	undiluted	DAKO		
ki-67	Monoclonal	Mouse	1:50	HIER (autoclave) Target Retrieval Solution, Citrate pH 6	DAKO	EnVision/HRP	undiluted	DAKO		
INSM1	Monoclonal	Mouse	1:300	HIER (autoclave) Target Retrieval Solution, Citrate pH 6	Santa Cruz	EnVision/HRP	undiluted	DAKO		

ACTH, adrenocorticotropic hormone; FSH, follicle-stimulating hormone; GH, growth hormone; LH, luteinizing hormone; TSH, thyroid-stimulating hormone; PIER, Proteolytic-Induced Epitope Retrieval; HIER, Heat-Induced Epitope Retrieval.

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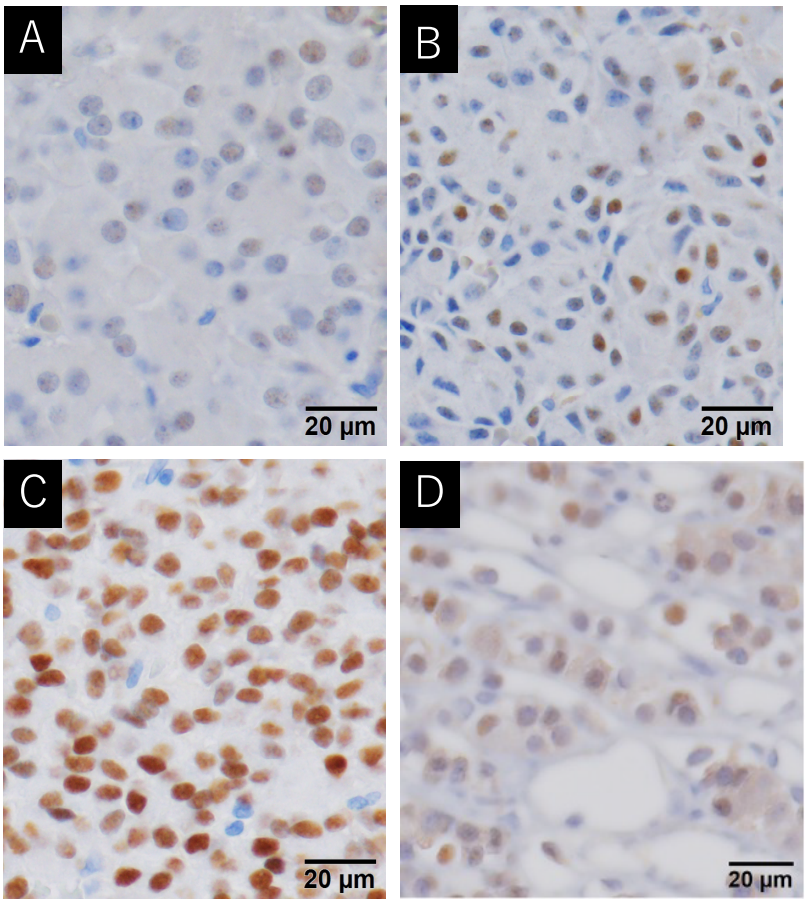

Fig. 1. Immunohistochemical analysis of INSM1 in illustrative PitNET samples. The results were interpreted as scores of 0 (negative), 1 (focally positive), 2 (frankly positive) points. (**A**) Case 5. Somatotroph tumor, score 1 (positive cell percentage: 3.5%). (**B**) Case 19. Corticotroph tumor, score 2 (positive cell percentage: 68%). (**C**) Case 30. Gonadotroph tumor, score 3 (positive cell percentage: 100%). (**D**) The majority of the normal pituitary cells were positive for INSM1.

(TPIT), pituitary-specific positive transcription factor 1 (Pit1), INSM1, and the epithelial marker CAM5.2, were also examined. Sources of the antibodies and staining protocols are indicated in Table 1.

The antigen retrieval process was performed using EnVision FLEX WASH BUFFER (1:20 diluted in DW; DAKO, Carpinteria, CA, USA) by autoclaving at 121°C for 10 min. The paraffin sections were immunostained with anti-INSM1 antibodies (A-8) SC-271408 (mouse, monoclonal, 1:300 diluted in 0.01 M phosphate-buffered saline; Santa Cruz Biotechnology, Dallas, TX, USA) at room temperature for 20 min. Next, treatment with EnVision/HRP (DAKO) as the secondary antibody was performed at room temperature for 20 min. The primary antibody was substituted with normal serum for the negative controls.

Based on the findings of the immunohistochemical analysis of the anterior pituitary hormones and transcriptional factors, including SF1, Pit1, and TPIT, the 35 PitNETs were classified into somatotroph tumors (STs), lactotroph tumors (LTs), thyrotroph tumors (TTs), corticotroph tumors (CTs), gonadotroph tumors (GTs), and unclassifiable tumors (UTs).

The Ki-67 labeling index (LI) was determined by examining the entire tissue section under high-power magnification (×400) after immunostaining. The number of cells stained positively with the Ki-67 antibody and the total number of tumor cells were counted in several representative fields containing more than 1,000 cells using e-Count (e-Path Co. Ltd., Kanagawa, Japan). The ratio was expressed as Ki-67 LI (%).

The results were interpreted as scores of 0 (negative) if positive staining was observed in fewer than 1% of the cells, 1 (focally positive) if positive staining was seen in 2–19% of the cells, 2 (frankly positive) if it was seen in >20% of the cells (Fig. 1) according to Rosenbaum *et al.* [10]. The normal pituitary tissues obtained during surgery were used as positive control. Although the non-tumorous part within the tumor tissue of the surgical specimen was determined to be normal tissue by the pathologists, the normal specimen used in this experiment contains some limitations.

This retrospective study was approved by the institutional review board of the International University of Health and Welfare (22-m-051).

Table 2. *Immunohistochemical findings of anterior pituitary hormones and transcriptional factors*

PitNET Type	Case no.	Sex	Age	Anterior pituitary hormones							Ki-67	LMWCK		Transcription factors			INSM1
				GH	PRL	TSH	ACTH	FSH	LH	αSU				Pit1	SF1	Tpit	
STs	Case 1	F	42	+	−	−	−	−	−	±	1>	+	Fb>70%	+	−	−	2
	Case 2	M	55	+	−	−	−	−	−	+	1>	+	Fb−	+	−	−	0
	Case 3	F	64	+	+	+	−	−	−	+	1>	+	Fb−	+	−	−	0
	Case 4	F	46	+	+	−	−	−	−	+	1>	+	Fb−	+	−	−	0
	Case 5	M	44	+	+	−	−	−	−	+	1>	+	Fb−	+	−	−	1
LTs	Case 6	M	63	±	+	−	−	−	−	±	2.3		N.D.	+	−	−	2
	Case 7	M	46	−	+	−	−	−	−	−	1.5		N.D.	+	−	−	1
	Case 8	F	68	+	+	−	−	−	−	−	1>		N.D.	+	−	−	0
	Case 9	M	61	−	+	−	−	−	−	−	1>		N.D.	+	−	−	0
	Case 10	M	47	−	+	−	−	−	−	−	1.7		N.D.	+	−	−	2
TTs	Case 11	F	58	+	−	+	−	−	−	+	1>		N.D.	+	−	−	2
	Case 12	F	47	+	−	+	−	−	−	+	1>		N.D.	+	−	−	2
	Case 13	M	69	+	−	+	−	−	−	+	1>		N.D.	+	−	−	2
	Case 14	F	67	+	±	+	−	−	−	+	1>		N.D.	+	−	−	2
	Case 15	M	40	+	+	+	−	−	−	+	2.6		N.D.	+	−	−	2
CTs	Case 16	M	44	−	−	−	+	−	−	−	1>	+	CC−	−	−	+	2
	Case 17	F	58	−	−	−	+	−	−	−	1>	+	CC−	−	−	+	2
	Case 18	F	59	+	+	−	+	−	−	−	1>	+	CC−	−	−	+	2
	Case 19	F	31	−	−	−	+	−	−	−	1>	+	CC−	−	−	+	2
	Case 20	F	44	−	−	−	+	−	−	−	2.2	+	CC−	−	−	+	2
	Case 21	F	48	−	−	−	−	−	−	−	1>		N.D.	−	−	+	1
	Case 22	F	40	−	−	−	−	−	−	−	1.1		N.D.	−	−	+	2
	Case 23	F	53	−	−	−	−	−	−	−	1		N.D.	−	−	+	2
	Case 24	F	78	−	−	−	−	−	−	−	1>		N.D.	−	−	+	2
GTs	Case 25	F	72	−	−	−	−	+	−	+	2		N.D.	−	+	−	2
	Case 26	M	81	−	−	−	−	+	−	+	1>		N.D.	−	+	−	0
	Case 27	F	44	−	−	−	−	+	−	−	1.5		N.D.	−	+	−	2
	Case 28	F	77	−	−	−	−	+	+	+	1>		N.D.	−	+	−	2
	Case 29	M	66	−	−	−	−	+	−	−	1		N.D.	N.D.	N.D.	N.D.	2
	Case 30	M	58	−	−	−	−	+	−	+	2.2		N.D.	−	+	−	2
	Case 31	M	75	−	−	−	−	+	−	−	1>		N.D.	−	+	−	2
	Case 32	M	64	−	−	−	−	+	−	+	2		N.D.	−	+	−	2
	Case 33	M	31	−	−	−	−	+	−	+	2		N.D.	−	+	−	2
	Case 34	F	76	−	−	−	−	−	−	−	1>		N.D.	−	−	−	0
UT	Case 35	M	66	−	±	+	−	+	−	+	1>		N.D.	+	+	−	1

ACTH, adrenocorticotropic hormone; αSU, alpha subunit; CC, Crooke's change; CTs, corticotroph tumors; Fb, fibrous bodies; FSH, follicle-stimulating hormone; GH, growth hormone; GTs, gonadotroph tumors; LH, luteinizing hormone; LMWCK, low molecular weight cytokeratin; LTs, lactotroph tumors; N.D., not determined; PitNET, pituitary neuroendocrine tumor; PRL, prolactin; STs, somatotroph tumors; TSH, thyroid-stimulating hormone; TTs, thyrotroph tumors; UT, unclassifiable tumor

III. Results

The samples were obtained from patients aged 31–78 years, with a mean age of 56.62 years (standard deviation: 13.69). The male:female ratio was 16:19. The 35 PitNETs were classified into 5 somatotroph tumors (STs), 5 lactotroph tumors (LTs), 5 thyrotoroph tumor (TTs), 9 corticotroph tumors (CTs), 10 gonadotroph tumors (GTs), and 1 unclassifiable tumor (UT).

Since the TTs collected in this study were confirmed to express growth hormone and prolactin, they could not be strictly classified as TTs but may possibly be classified as mature plurihormonal Pit1-positive tumors according to the World Health Organization (WHO) 2022. The nuclei of 28 of the 35 PitNETs (80%) were immunohistochemically positive for INSM1.

The staining for each histological subtype (WHO classification of endocrine tumors: WHO, 2022) of PitNETs was as follows: STs: score 0 = 3/5, score 1 = 1/5, score 2 = 1/5; LTs: score 0 = 2/5, score 1 = 1/5, score 2 = 2/5; TTs: score 2 = 5/5; CTs: score 1 = 1/9, score 2 = 8/9; GTs: score 0 = 2/10, score 1 = 0/10, score 2 = 8/10 and UT: score 1 = 1/1.

Findings of the immunohistochemical analysis of the anterior pituitary hormones and transcriptional factors are presented in Table 2 and Fig. 1.A, B, and C. In the normal pituitary tissue, the majority of the pituitary cells were positive for INSM1, as shown in Fig. 1. D. The non-tumor

areas of surgical specimens were stained as normal tissue, and a similar trend of staining was observed in several specimens.

IV. Discussion

INSM1 is a transcription factor that induces differentiation in neuroendocrine cells or tumor cells in various organs, and many reports have shown its usefulness in diagnosis and treatment, including molecular-targeted therapy. In various tumors, such as thoracic tumors, laryngeal neuroendocrine carcinomas, and pancreatic neuroendocrine tumors, differences in sensitivity, specificity, and staining have been reported compared with classical markers, such as synaptophysin, chromogranin, and CD56 [5, 14, 12].

In the normal anterior pituitary gland, INSM1 is expressed along with various hormones, suppressing inappropriate differentiation [13]. PitNETs are derived from the clonal expansion of mutated somatic cells [7]. Historically, PitNETs were classified based on hormone secretion. However, transcription factors were included in the classification category in the fourth edition of the WHO classification of endocrine organ tumors, published in 2017. The new 2022 WHO classification of endocrine tumors follows this trend. The 2022 WHO classification categorized tumors with a concept of cell lineage based on transcription factors and other biomarkers.

Immunohistochemical staining of transcription factors, such as Pit1, TPIT, SF1, ERα, and GATA3, complements this classification [1].

In a comparison of INSM1 expression in PitNETs with conventional markers, such as synaptophysin, chromogranin, and CD56, which are typically expressed in neuroendocrine cells, INSM1 was found to be significantly more sensitive than conventional markers [9]. Additionally, INSM1 was negative in malignant head-and-neck-region tumors that were not derived from neuroendocrine cells. Hence, INSM1 has high sensitivity and specificity as a marker of neuroendocrine cell origin [9].

However, reports of its expression in PitNETs are sporadic. The positivity rate of INSM1 in PitNETs in this study was as high as those reported by Rooper *et al.* [9] and Rosenbaum *et al.* [10]. INSM1 is expressed in normal anterior pituitary cells in mice, showing its involvement in maintaining the characteristics of neuroendocrine tumors of the anterior pituitary [13].

In this study, all cases with attenuated or negative expression of INSM1 were densely granulated STs with a low Ki-67 LI. The INSM1 positivity rate for LT and ST in our study was 50% (5/10), whereas that for the other tumor types was 92% (23/25).

The molecular mechanism of the lower positivity rate for LT and ST should be further investigated using a larger series.

INSM1 staining can be seen in normal pituitary cells and PitNETs. INSM1 is involved in the induction of differentiation into hormone-secreting cells in the normal pituitary gland [13]. Our study suggested the possible role of INSM1 in anterior pituitary cell-derived tumor development into various hormone-secreting tumors.

In the pituitary gland, INSM1 expression is initiated during endocrine cell differentiation and continues during development, even into adulthood, controlling cell differentiation and suppressing inappropriate gene expression [13].

We investigated the expression of INSM1 in PitNETs using immunohistochemical analysis. Similar to the normal pituitary gland, most PitNETs maintained the expression of INSM1, showing that they could maintain the characteristics of neuroendocrine tumors of the anterior pituitary.

In conclusion, our findings suggest that analysis of expression of INSM1 is useful in the diagnosis of PitNETs.

V. Conflicts of Interest

The authors declare that there are no conflicts of interest.

VI. Acknowledgments

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