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Regular Article

Immunohistochemical Analyses of Mammalian Target of Rapamycin (mTOR) Expression in Pituitary Neuroendocrine Tumors (PitNETs): mTOR as a Therapeutic Target for Functional PitNETs

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Current therapeutic modalities for pituitary neuroendocrine tumors (PitNETs) include medication, surgery, and radiotherapy. Some patients have tumors that are refractory to current modalities. Therefore, novel treatment options are needed for patients with intractable diseases. Consequently, we examined the pathological data of PitNETs to study medical therapies. We retrospectively studied 120 patients with histologically diagnosed PitNETs. We used the data for the histopathological examination of hormones, such as growth hormone (GH), prolactin (PRL), adrenocorticotropic hormone, thyroid stimulating hormone, luteinizing hormone, follicle-stimulating hormone, and α-subunit, together with the immunohistochemical studies of the phospho-mammalian target of rapamycin (mTOR), cytokeratin (CAM5.2), somatostatin receptor (SSTR) type 2 and 5, Pit-1 (POU1F1/GHF-1), steroidogenic factor-1 (SF-1), and Tpit. GH-, PRL-, and SSTR5-immunopositive PitNETs had significantly higher percentage of mTOR-positivity, compared with GH-, PRL-, and SSTR5-immunonegative Pit NETs. Our results show that activation of the AKT/phosphatidylinositol-3-kinase pathway, including mTOR activation, might be related the development of PitNETs, especially GH- and PRL-producing PitNETs. Thus, mTOR is a potential target for treating functional PitNETs.

Key words: pituitary neuroendocrine tumor, immunohistochemistry, phospho-mammalian target of rapamycin (mTOR)

I. Introduction

Pituitary neuroendocrine tumors (PitNETs), formerly known as pituitary adenomas, are common intracranial neoplasms [14]. Current therapeutic modalities include medication, surgery, and radiotherapy [1, 15]. While some patients are cured or their symptoms controlled by these therapies, a considerable proportion exhibits tumors that are refractory to current modalities [1]. Novel options are therefore required for these intractable patients, and therapy targeting signal transduction pathways that promote tumor growth may represent a promising approach for patients with refractory tumors.

Although our understanding of the pathophysiology of

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Table 1. *Primary antibodies concerning pituitary hormones and transcription factors, and marker proteins. These are diluted in 0.01 M phosphate-buffered saline.*

Primary antibody					
	Antibodies	Nature of antibodies	Dilution rate	Company	Epitope Retrival
Hormones	GH	polyclonal(rabbit)	*1:400	DAKO(Carpinteria, CA, U.S.A.)	
	PRL	polyclonal(rabbit)	1:400	DAKO (Carpinteria, CA, U.S.A.)	
	ACTH	monoclonal(mouse)	1:400	DAKO (Carpinteria, CA, U.S.A.)	
	TSH	monoclonal(mouse)	1:200	DAKO (Carpinteria, CA, U.S.A.)	
	LH	monoclonal(mouse)	1:200	IMMUNOTECH/MBL (Minato-ku, Tokyo, Japan)	
	FSH	monoclonal(mouse)	1:200	IMMUNOTECH/MBL (Minato-ku, Tokyo, Japan)	
	α-SU	polyclonal(rabbit)	1:1000	NIDDK (Rockville Pike, Bethesda, U.S.A.)	
Transcription factors and Marker proteins	Phospho-mTOR	polyclonal(rabbit)	1:100	Cell Signaling technology (Danvers, MA, U.S.A.)	water bath to 99 °C for 40 min
	CAM5.2	monoclonal(mouse)	1:2	BD (Franklin Lakes, NJ, U.S.A.)	water bath to 99 °C for 40 min
	SSTR2	monoclonal(rabbit)	1:100	Epitomics (Burlingame, California, U.S.A.)	autoclave to 121 °C for 10 min
	SSTR5	monoclonal(mouse)	1:400	Epitomics (Burlingame, California, U.S.A.)	autoclave to 121 °C for 10 min
	SF-1	monoclonal(mouse)	1:100	Invitrogen (Waltham, Massachusetts, U.S.A.)	autoclave to 121 °C for 10 min
	Pit-1	monoclonal(mouse)	1:100	Santa Cruz (Dallas, Texas, U.S.A.)	water bath to 99 °C for 40 min
	Tpit	(Rabbit)	1:300	Dr	autoclave to 121 °C for 10 min

PitNETs has expanded, the precise pathogenetic mechanisms of PitNETs are yet to be elucidated. The activation of the AKT/phosphatidylinositol-3-kinase pathway, including the mammalian target of rapamycin (mTOR) activation, is considered to be one of the intracellular pathways involved in PitNET development [1, 4, 14]. In this study, we present our analysis of the pathological data on PitNETs, focusing particularly on mTOR expression, with significant indicative results for medical therapeutics.

II. Materials and Methods

Case materials

An unselected series of patients (68 male and 72 female patients) with PitNETs treated at Teikyo University Hospital and the International University of Health and Welfare Mita Hospital were enrolled in this study. The age of the patients ranged from 16 to 87 years, with a mean age of 52.4 years (standard deviation [SD]: 17.6). The details of their histopathological data were collected and analyzed. The Institutional Review Board approved this clinical study (license number 5-21-53), and written informed consent was obtained from the patients for their endocrinological and pathological data. The pathological diagnosis of PitNETs was established by two independent pathologists (C.I. and R. Y. O.) who were blinded to the patients' data. Consecutive specimens were used to compare pituitary hormone immunostaining, transcriptional factors, and marker proteins. Positive results were determined as + for 1–25%, 2+ for 26–50%, 3+ for 51–75%, and 4+ for >75% immunopositivity, respectively.

Histopathological examination

Immunohistochemistry of anterior pituitary hormones

Deparaffinized sections were washed with running tap water, rinsed with distilled water, and washed 0.01 M phosphate-buffered saline (PBS). They were incubated in 1% hydrogen peroxide solution for 10 min at room temperature (RT) to block endogenous peroxidase.

After washed with running tap water and washed three times with Wash buffer (Wash Buffer, Concentrate 20x (DAKO, Carpinteria, CA, U.S.A.)). They were incubated with primary antibodies show in Table 1, overnight at 4°C and with ACTH and TSH antibodies for 20 min at RT. Further, they were washed three times with Wash buffer and incubated with secondary antibodies for 45 min at RT (for ACTH and TSH for 20 min at room temperature). After they were washed three times with Wash buffer, Immunoreactions were visualized using an Envision FLEX/HRP kit (DAKO, Carpinteria, CA, U.S.A.). The sections were then washed with running tap water for 10 min. After dehydration in 70% EtOH, 80% EtOH, 90% EtOH, 95% EtOH, and 100% EtOH and processing in xylene, the sections were cover-slipped with Entellan new. Images were collected using the Olympus BX50 light microscope.

Transcription factors and maker proteins

Immunohistochemistry

Deparaffinized sections were washed with running tap water, rinsed with distilled water, and washed PBS. Deparaffinized sections were washed with running tap water. Antigen retrieval was performed by Water Bath or Autoclave, as shown in Table 1. They were left at RT for 20 minutes. After washing with running tap water, they were incubated in 1% hydrogen peroxide solution for 10 min at RT to block endogenous peroxidase. They were washed with running tap water and washed three times with Wash buffer they were incubated with primary antibodies shown in Table 1 overnight at 4°C. Especially for SSTR2 and SSTR5, they were washed with linker solution (Rabbit LINKER (DAKO, Carpinteria, CA, U.S.A.)). Next, they were incubated with secondary antibodies (Table 2) for 45 min at RT (for SSTR2, SSRT5 and mTOR for 20 min at RT) After, they were washed three times with Wash buffer, Immunoreactions were visualized using an Envision FLEX/HRP kit. The sections were then washed with running tap water for 10 min. After dehydration in 70% EtOH, 80% EtOH, 90% EtOH, 95% EtOH, and 100% EtOH and

Table 2. *Secondary antibodies. These are diluted in BSA-phosphate-buffered saline.*

Secondary antibody			
	Product	Dilution rate	Company
Anti-mouse IgG	HRP-Linked F (ab') 2 Fragment Donkey	1:100	Cytiva (Shinjuku-ku ,Tokyo, Japan)
Anti-rabbit IgG	HRP-Linked F (ab') 2 Fragment Sheep	1:400	Cytiva (Shinjuku-ku ,Tokyo, Japan)
ACTH,TSH	Envision FLEX/HRP kit	-	DAKO (Carpinteria, CA, U.S.A.)

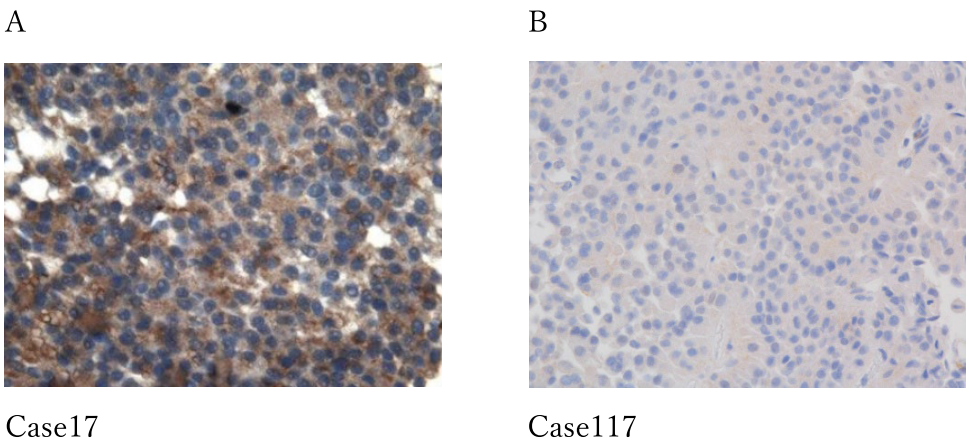

Fig. 1. **a**; Illustrative mTOR immunopositive case, **b**; Illustrative mTOR immunonegative case.

processing in xylene, the sections were cover-slipped with Entellan new. Images were collected using the Olympus BX50 light microscope.

The specificity of primary antibodies used in this study was confirmed in the previous studies [4, 6–10, 12, 13, 18].

Negative control samples involved replacement of the primary antibody with normal sera.

Statistical analyses

Statistical analyses were performed using Excel 2021 (Microsoft, Redmond, WA, U.S.A.) and the chi-squared test. Values are shown as mean ± S.D., and $p < 0.05$ was interpreted to be statistically significant.

III. Results

In this study, 85 out of 120 patients with PitNETs were found to be immunopositive for mTOR, while 35 were immunonegative. Representative mTOR positivity and negativity results are shown in Fig. 1. mTOR was immunopositive in the cytoplasm. The pathological data of the PitNETs are shown in Table 3.

The mean patient age at the time of surgery was 51.4 ± 17.2 years for the mTOR-positive immunostaining group versus 54.8 ± 18.2 years for the mTOR-negative immunostaining group. No significant differences in mean age were found between the two groups ($p = 0.36$).

The ratio of mTOR positivity to negativity was significantly higher in female patients than in male patients ($p < 0.05$). Among male patients, 64% were mTOR-positive, and 36% were mTOR-negative. Among female patients, 81% were mTOR-positive, and 19% were mTOR-negative.

Growth hormone (GH)-immunopositive specimens and prolactin (PRL)-immunopositive specimens had high percentage of significant mTOR-positivity (both, $p < 0.05$), compared with GH-, PRL-, and SSTR5-immunonegative Pit NETs.

Among GH positive PitNETs, 64% were mTOR-positive. Among GH negative PitNETs, 10% were mTOR-positive. Among PRL positive PitNETs, 88% were mTOR-positive. Among PRL negative PitNETs, 64% were mTOR-positive.

None of the other hormone-secreting PitNETs (ACTH, FSH, LH, and TSH) correlated with mTOR positivity.

Of SSTR5 positive PitNETs, 82% were mTOR-positive, whereas of SSTR5 negative PitNETs, 62% were mTOR-positive. mTOR immunopositivity was significantly more frequently observed in SSTR5 immunopositive tumors than in immunonegative tumors ($P < 0.05$). 75% of SSTR2 positive PitNETs were mTOR-positive, and 63% of SSTR2 negative PitNETs were mTOR-positive. Other transcription factors and marker proteins (SSTR2, Pit-1, Tpit, CAM5.2, and SF-1) including SSTR2 were examined in various cases; however, none correlated with mTOR immunopositivity.

Negative control studies with substitution of pituitary antibodies with normal sera showed no positive stainings.

IV. Discussion

mTOR is a molecule without organ specificity. It is expressed in the cytoplasm and activated by phosphorylation. Maruska KP *et al.* showed mTOR expression in nor-

Table 3. *Immunohistochemical staining results*

	M/F	年齢	mTOR	GH	PRL	ACTH	FSH	LH	TSH	αSU	SSTR2	SSTR5	CAM5.2	pit-1	SF1	Tpit
1	F	63	−	−	−	−	2+	−	−	−	−	−				
2	M	41	−	−	−	−	4+	−	−	−	−	−				
3	F	74	−	−	−	−	+	−	−	+	−	−				
4	M	33	+	−	−	−	−	−	−	−	−	−			+	
5	F	29	+	−	3+	−	−	−	−	−	−	−	−			
6	M	64	+	−	−	−	−	−	−	−	2+	−			+	
7	M	39	−	+	−	−	−	−	−	−	3+	3+	−	−		
8	M	54	+	−	−	−	+	−	−	+	2+	−				
9	M	30	+	+	−	−	−	−	−	−	2+	3+				
10	M	74	+	−	−	−	3+	−	−	+	2+	−				
11	M	63	−	−	−	−	−	−	−	+	−	−			+	
12	F	61	+	4+	2+	−	−	−	−	+	3+	2+	+			
13	M	53	+	4+	−	−	±	−	−	4+	3+	3+	+			
14	M	60	−	−	−	−	−	−	−	−	−	−				
15	F	28	−	−	−	−	±	−	−	−	−	−	+			
16	F	35	−	−	−	−	4+	−	−	−	−	−				
17	M	73	−	−	−	−	−	−	−	+	−	−				
18	M	39	+	4+	−	−	−	−	−	−	3+	2+	+			
19	F	41	+	+	−	−	−	−	−	−	3+	3+	+			
20	M	46	−	−	−	−	−	−	−	−	3+	3+				
21	M	66	−	−	−	−	4+	−	−	−	2+	−				
22	M	66	−	−	−	−	−	−	−	+	2+	2+				
23	M	62	+	+	+	−	−	−	−	−	3+	3+	+			
24	F	60	−	−	−	−	±	−	−	−	2+	−				
25	M	49	−	−	+	−	−	−	−	−	−	2+				
26	F	30	+	−	3+	−	−	−	+	4+	3+	−	−			
27	F	40	−	−	−	−	±	−	−	+	2+	−	-	+		
28	M	50	+	−	−	−	4+	−	−	2+	3+	−		+		
29	M	78	+	−	−	−	−	+	−	+	2+	−		+		
30	M	51	+	4+	−	−	−	−	−	−	3+	3+	+			
31	M	53	+	−	−	4+	−	−	−	−	−	2+	+			
32	M	30	+	4+	+	−	−	−	+	3+	3+	3+	+			
33	M	19	−	−	−	−	4+	−	−	2+	3+	−				
34	F	62	+	−	−	4+	−	−	−	−	−	3+	+			
35	M	70	+	−	−	−	2+	+	−	3+	−	−				
36	F	59	+	3+	+	−	−	−	−	+	3+	3+	+			
37	M	58	+	−	−	−	2+	−	−	2+	2+	−				
38	M	31	+	4+	2+	+	−	±	±	3+	3+	2+	+			
39	M	48	+	4+	2+	4+	±	−	−	4+	−	2+	+			
40	F	68	+	3+	2+	−	−	−	−	−	−	−	3+	−		
41	M	41	+	4+	3+	−	±	±	−	2+	3+	3+	+			
42	M	58	+	−	−	−	−	−	−	3+	−	−				
43	M	87	−	−	−	−	−	−	−	−	2+	−		+		
44	M	26	+	−	−	−	−	−	−	−	+	−	+			
45	F	74	+	4+	2+	−	−	−	+	3+	3+	2+	+			
46	F	69	+	−	−	−	−	+	−	+	2+	−		+		
47	F	30	+	−	−	−	−	−	+	4+	+	2+	+	+	+	
48	M	47	+	−	−	−	−	−	−	−	3+	−		+	+	+
49	F	61	+	−	−	−	2+	−	−	3+	2+	−				
50	M	51	+	−	−	−	+	−	−	−	−	−		+	+	
51	M	49	+	−	−	−	−	−	−	+	3+	−		+	+	
52	M	52	+	−	−	−	−	−	−	+	2+	−		+	+	
53	M	65	+	4+	2+	−	−	−	−	2+	3+	3+	+			
54	M	62	−	−	−	−	+	+	−	+	2+	−		+	+	
55	F	16	+	−	4+	−	−	−	−	−	−	2+				
56	F	41	+	+	−	−	−	−	−	−	3+	−	−	+		
57	M	53	+	−	−	−	2+	+	−	2+	2+	−				
58	M	43	+	−	−	−	+	+	−	2+	2+	2+				
59	F	34	+	−	−	−	−	−	−	−	−	−	−	+	+	+
60	M	52	+	−	−	4+	−	−	−	−	−	2+	+			
61	F	61	+	−	+	−	−	−	+	4+	3+	2+	−			
62	M	31	+	−	4+	−	−	−	−	−	2+	−				
63	F	61	+	4+	2+	−	−	−	−	2+	3+	2+	+			
64	M	35	+	4+	+	−	−	−	−	+	3+	3+	+			
65	M	36	+	4+	+	−	−	−	3+	+	3+	3+	−	+	+	+
66	M	80	+	−	−	−	+	−	−	+	2+	−	+	+	−	
67	F	83	+	−	−	−	−	−	−	−	+	−		+	−	−
68	F	50	+	−	−	±	−	−	−	−	−	−	+	−	−	−
69	F	47	+	2+	±	−	−	−	−	2+	3+	3+	+	+		+
70	F	59	+	−	+	+	−	−	−	−	−	−	+	+	−	+
71	F	31	+	2+	4+	−	−	−	−	−	−	3+	+			
72	M	42	+	−	−	−	−	−	+	+	3+	3+	+	+	−	
73	M	75	−	−	−	−	−	−	−	−	2+	−	+	−	−	−
74	M	51	+	−	−	−	+	−	−	2+	3+	2+				
75	F	77	+	−	−	±	−	−	−	−	−	−				
76	M	70	+	−	+	−	2+	−	−	2+	3+	−		−	−	+
77	M	16	+	−	−	4+	−	−	−	−	−	−	+			+
78	F	68	+	−	−	−	+	−	−	2+	2+	−		+	+	
79	F	74	+	4+	±	−	−	−	−	+	3+	3+	+	+		
80	M	78	+	−	−	−	−	−	−	+	2+	−		−	+	
81	M	54	+	−	−	−	−	−	−	−	2+	−		−	+	−
82	F	46	+	−	−	−	−	−	−	+	2+	−	−	−	−	
83	F	56	+	−	−	−	−	−	−	+	3+	−		−	−	−
84	F	64	+	−	−	−	−	−	−	2+	2+	2+		+	+	−
85	F	69	+	−	−	−	−	−	−	−	−	−	+	−	−	+
86	F	74	+	+	+	−	−	−	−	−	3+	3+	+	+		
87	F	71	+	4+	2+	−	−	−	−	2+	3+	3+	+	+		
88	M	65	+	−	−	−	±	−	−	+	+	−			+	
89	F	74	+	−	−	−	+	−	−	2+	2+	−	−	−	+	
90	M	45	+	−	−	−	−	−	−	±	3+	−		−	+	
91	M	44	−	−	−	−	−	−	−	+	3+	−		−	+	
92	M	66	−	−	−	−	−	−	−	2+	2+	−		−	+	
93	M	58	+	−	−	2+	−	−	−	−	2+	−	+			+
94	M	61	+	+	2+	−	−	−	−	±	2+	3+	+			
95	M	56	−	−	4+	−	−	−	−	−	−	2+	+	+	−	
96	M	44	+	4+	+	−	−	−	−	3+	3+	3+	+	+	−	
97	F	25	+	−	3+	−	−	−	−	−	−	3+	−	−	+	
98	F	25	+	−	4+	−	−	−	−	−	−	+	+	+		
99	M	28	−	−	−	−	3+	−	−	+	+	−	+	−		
100	M	28	+	3+	±	−	−	−	−	+	3+	2+	+	+	−	
101	F	74	+	4+	−	−	−	−	+	2+	3+	3+	+	+	−	
102	F	61	+	−	−	+	−	−	−	−	−	−	+	−	−	+
103	F	51	+	−	−	+	−	−	−	−	−	−	+	−	−	+
104	F	29	−	+	−	−	−	−	−	−	2+	3+	+			
105	M	47	−	−	−	−	+	−	−	+	2+	−		−	+	
106	M	81	+	−	−	−	−	2+	−	+	+	−		−	+	
107	M	67	−	−	−	−	−	−	−	3+	−	−		+	+	
108	M	70	−	−	−	−	−	−	−	+	2+	−		−	+	
109	M	65	−	−	−	−	−	−	−	+	3+	−			+	
110	F	20	+	−	3+	−	−	−	−	+	−	+	+			
111	F	22	+	−	3+	−	−	−	−	−	−	3+	+			
112	M	26	+	−	3+	−	−	−	−	−	−	2+	−		−	
113	M	51	−	−	−	−	−	−	−	+	2+	−			+	
114	F	39	+	−	−	±	−	−	−	−	−	−	+	−	−	+
115	M	35	−	2+	4+	−	−	−	−	−	−	3+	+	+		
116	M	18	−	−	−	3+	−	−	−	±	−	−	+	−	−	+
117	F	72	−	−	−	−	−	−	−	−	−	−	+	−	−	+
118	M	60	−	−	4+	−	−	−	−	−	−	2+	+	+	−	
119	F	86	−	−	−	−	+	+	−	+	2+	−	−		+	
120	M	79	−	−	−	−	−	−	−	2+	3+	−	+		+	

mal pituitary cells using reverse transcription polymerase chain reaction and *in situ* hybridization methods. [11]

The activation of the AKT/phosphatidylinositide-3-kinase pathway, including activation of mTOR, is common in human neoplasms. This signal transduction cascade involves numerous crucial cellular functions, including cell cycle regulation, cell survival, cell growth, protein synthesis, and cellular metabolism. mTOR activation has been implicated in other human cancers, and preclinical studies have demonstrated the therapeutic effects of targeting mTOR in PitNETs [1, 2, 4, 5].

Basal activation of mTOR downstream of constitutive AKT signaling was observed in rat GH3 cells. Functionally, rapamycin as well as everolimus (both mTOR inhibitors) induce G1 growth arrest within 24 h, an effect associated with reduced cellular proliferation [16]. The inhibition of mTOR also radiosensitizes rat GH3 cells such that radiation in combination with rapamycin or everolimus reduces cellular viability more effectively than radiation alone [15].

In animal experiments, hyperactivation of mTOR signaling was observed in estrogen-induced rat pituitary tumors, and rapamycin blocked tumor development [3].

Currently, the medications that have shown efficacy in controlling hormonal abnormality and reducing the size of PitNETs are dopamine agonists for prolactin-secreting PitNETs [3] and somatostatin analogs for growth hormone-secreting PitNETs [1, 14].

Somatostatin is a peptide with potent and broad antisecretory actions, and serves as an invaluable drug target for the pharmacological management of PitNETs and neuroendocrine tumors [3]. In recent years, somatostatin analogs, dopamine agonists, and their combination therapies with inhibitors of mTOR targets have been reported [3, 19].

The AKT/phosphatidylinositide-3-kinase pathway is involved in SSTR antiproliferative signaling, and AKT overexpression blocks the antiproliferative activity of somatostatin analogs in pituitary tumor cells. Therefore, inhibition of this pathway is expected to improve the efficacy of somatostatin analog [3, 14, 17].

Adding rapamycin to octreotide (somatostatin analog) treatment potentiates its antiproliferative effect in AtT-20 cells (experimental ACTH-producing pituitary tumors) and human non-functioning PitNETs *in vitro* [2].

In vitro studies using experimental rat GH3 cells have demonstrated that both cabergoline (a dopamine agonist) and everolimus inhibited GH3 cell proliferation and PRL secretion as single agents, and a synergistic effect on the inhibition of PRL was noted with their combination treatment [19].

In clinical trials, combination therapy with everolimus and octreotide in patients with advanced neuroendocrine tumors has been reported to have promising antitumor activity [16, 17].

In one case report, everolimus, in addition to cabergoline, decreased PRL levels and tumor regression after 5 months of treatment for prolactinomas [19].

In this study, mTOR immunopositivity was frequently observed, particularly in GH- and PRL-immunopositive cases. Therefore, although further investigation is necessary pharmacological inhibition of the mTOR pathway may serve as an effective therapeutic strategy for treating PitNETs, especially GH- and PRL-immunopositive tumors.

The correlation between mTOR- and SSTR 5-positive cases may also indicate the possible concomitant use of mTOR inhibitors and somatostatin analog therapy.

Pharmacological inhibition of the mTOR pathway, using a combination of pre-existing therapeutics, may be an effective therapeutic strategy for treating PitNETs; however, further research is required.

V. Conflicts of Interest

The authors declare that there are no conflicts of interest.

VI. Acknowledgments

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VII. References

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