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Original

Rab3a and Rab27b Expression in Nonfunctioning Pituitary Adenomas

Akiko Sasaki[1], Katsuhiko Takahashi[2], Gou Yamamoto[3], YukoTsunoda[4], Takumi Abe[1] and Tetsuhiko Tachikawa[3]

Abstract: Patients with nonfunctioning pituitary adenoma (NFPA) have normal circulating levels of anterior pituitary hormones. Here we examined the expression of exocytic trafficking regulators, Rab27b and Rab3a, in surgically resected pituitary adenoma tissues by immunohistochemical (IHC) analysis using anti-Rab27b and anti-Rab3a antibodies. Among the examined tissues, just over half of the null-cell adenomas and one-third of the gonadotropin-producing adenomas were immunonegative for both Rab27b and Rab3a. However, no Rab-negative samples were observed among the functioning adenomas. These results suggested that downregulated Rab protein expression in anterior pituitary endocrine cells could underlie, at least in part, the hormone-secretion defects of nonfunctioning adenoma cells. Rab27b, Rab3a, and their cellular regulators might therefore be promising pathological markers of patients with NFPA.

Key words: hormone secretory system, nonfunctioning pituitary adenoma, Rab3a, Rab27b

Introduction

Nonfunctioning pituitary adenomas (NFPA) lack the hormone-related clinical signs and symptoms characteristic of most pituitary tumors. Comprising around 30% of all pituitary adenomas, NFPA do not manifest clinically in easily recognizable pituitary hypersecretory syndromes and consequently, the associated tumors are often large when finally detected. Headaches and visual-field deficits due to compression of the optic nerve is therefore a common presentation for patients with NFPA and most preliminary scan results in these patients reveal neuro-ophthalmological or neurological symptoms[1-3]. Early detection of adenomas leads to more effective treatments, thus the challenge for better prognoses in NFPA patients is identifying testable peculiarities of this disease.

Patients with NFPA have normal circulating levels of anterior pituitary hormone.

[1] *Department of Neurosurgery, Showa University School of Medicine,* 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8666, Japan.
[2] *Department of Biological Chemistry, Showa University School of Pharmaceutical Sciences.*
[3] *Department of Oral Pathology, Showa University School of Dentistry.*
[4] *Department of Breast Surgery, Showa University School of Medicine.*

Gonadotropin-producing adenoma is clinically classified among the NFPA, because most cases present with normal levels of gonadotropin. In contrast, cases of functioning pituitary adenomas including prolactinoma and growth hormone (GH)-producing adenoma show increased circulating levels of prolactin (PRL) and GH, respectively[4]. It was therefore proposed that gonadotropin-producing adenoma cells might have defective hormone-secretion mechanisms, although this has yet to be confirmed experimentally[5–9].

Gonadotropin-producing adenoma cells contain vesicles that transport follicle-stimulating hormone (FSH)-β and lutenizing hormone (LH)-β[10–11]. The Rab family of GTPase proteins regulates vesicular transport during all stages of intracellular protein trafficking, including the multiple and complex pathways of endocrine cells[12]. Pituitary endocrine cells express several Rabs including Rab27b and Rab3a. Rab27 regulates the delivery of granules to the exocytic site and the Rab27b isoform has been localized on pituitary endocrine granules[13]. Izumi's group reported tissue-specific expression of Rab27b in C57BL/6J mice, including in the brain and pituitary, and that Rab27b was differentially expressed in the various pituitary cell types that secrete different hormones[10, 14]. Rab3 proteins also have been associated with secretory vesicles, and several studies showed coexpression of Rab27b and Rab3a in secretory neuron cells[15]. However, Rab3a-deficient synapses displayed an enhanced rundown of synaptic transmission, implicating an inhibitory role for Rab3a in vesicle fusion[19]. It is therefore possible that Rab3 and Rab27 have different functions on secretory vesicles[16–19]. This study investigated the pathological significance of Rab proteins in human pituitary adenomas. We examined the expression of both Rab27b and Rab3a in surgically dissected pituitary adenomas by immunohistochemical (IHC) analysis.

Materials and Methods

Subjects

The study comprised 74 patients who underwent surgical resection for pituitary adenoma by T. Abe's cohort at Showa University Hospital from 1999 to 2008[20]. The diagnoses included two types of NFPA [gonadotropin-producing (Gn) adenoma and null-cell (Null) adenoma] and two types of functioning adenomas (PRL-producing adenoma and GH-producing adenoma), as follows: 16 Gn tumors, 23 Null adenomas, 17 GH-producing adenomas, and 18 PRL-producing adenomas (Table 1)[21]. The Showa University Ethics Review Board approved the study protocol.

Immunostaining

Sections of the tumor samples were deparaffinized, washed, and then incubated in 0.3% H_2O_2 for 10 min to block endogenous peroxidase activity. The sections were then incubated in nonspecific blocking reagent (DAKO X0909) for 5 min to block nonspecific staining, before the primary antibody incubations in mouse anti-Rab3a monoclonal antibody (BR-039, Innogenetics, Gent, Belgium) diluted 1:100 in Dako Antibody Diluent (DAKO S0809)

Table 1. Clinical laboratory findings in 74 patients with pituitary adenoma

		Nonfunctioning		Functioning	
		Null cell adenoma	Gonadotropin adenoma	GH adenoma	PRL adenoma
n		23	16	17	18
Age		61.2 (44–79)	53.8 (23–67)	43 (26–71)	38.4 (21–74)
Sex (male / female)		13 / 10	10 / 6	9 / 8	5 / 13
Serum hormone level (mU / l)	LH	5.3±4.2 (< 1.0–15.1)	4.4±4.0 (< 1.0–7.3)	8.6±6.9 (1.2–20.9)	5.9±7.3 (< 1.0–28.8)
	FSH	20.54±13.2 (3.3–36.1)	14.8±25 (1.0–96.8)	23.8±24.2 (4.6–83.1)	6.2±3.2 (1.3–11.2)
	GH	0.3±0.3 (0.1–0.7)	0.25±0.2 (0.1–0.7)	18.8±19.5 (1.5–51.0)	2.1±4.5 (0.1–16.3)
	PRL	15.4±10.0 (5.4–27.8)	19.4±34.2 (1.9–20.2)	18.8±18.3 (5.8–46.6)	766.2±927.5 (10.1–2860.2)

All values represent mean±SD (range)

for 30 min at room temperature. The sections were finally incubated for 30 min in HRP-labeled anti-mouse secondary antibody (K4007, Dako, Glostrup, Denmark) and counter-stained with Tissue-Tek hematoxylin. No-primary-antibody negative controls were run on parallel sections.

Immunofluorescence

Some of each resected tissue was also embedded in OCT compound (Sakura, Torrance, CA) and then snap frozen with isopentane cooled in liquid nitrogen. Frozen sections were cut at a thickness of 5 μm and fixed in 10% formaldehyde. These sections were again incubated in 0.3% H_2O_2 for 5 min to remove endogenous peroxidase activity and then in the Dako nonspecific blocking reagent for 5 min. Sections were then incubated with goat anti-Rab27b polyclonal antibody (sc-22991, Santa Cruz Biotechnology, CA) for 1 h at room temperature, followed by FITC-conjugated anti-goat IgG (F0250, Dako) as a secondary antibody in a humid chamber at 37°C for 30 min. The same sections were then washed thoroughly before incubation with mouse anti-Rab3a monoclonal antibody (BR-039, Innogenetics) at 37°C for 1 h, and then a TRIC-conjugated anti-mouse lgG (F0270, Dako) as secondary antibody for 30 min. Sections were systematically counterstained with Bisbenzimide H33342 (Hoechst 33342), and then viewed on a Nikon A1 confocal microscope.

Immunoreactivity grading was based on the German Immunoreactive score[22]. Staining intensity in the cytoplasm was rated on a scale from 0 to 3, with 0 being no staining at all, 1 weak staining, 2 moderate, and 3 strong staining.

For statistical purposes, two groups were formed: negative immunoreactivity was Negative (scale 0); weak and moderate and strong immunoreactivity were Positive (scale 1-3).

Statistical analysis

Characteristics between pituitary adenomas were compared statistically using the Fisher exact test for categorical variables. Results are expressed as mean +/− SD and statistical significance was set at $P < 0.01$.

Results

The relationship between Rab27b/Rab3a expression and pituitary adenoma pathology was investigated in this study by IHC analysis of NFPA and other pituitary adenoma samples. Hematoxylin/eosin (HE)-stained sections showed the papillary type cells that are typical of a pituitary adenoma in both Null and Gn adenomas (Fig. 1A, B). PRL adenomas displayed a diffuse-type cell pattern (Fig. 1C), while GH adenoma cells were acidophilic (Fig. 1D). We then examined Rab3a expression in these human pituitary adenoma tissues (Fig. 1E-H). A negative control was included in each run (obtained by omitting the primary antibody). Nonspecificity of Rab3a was not expressed (Fig. 1I-L). We examined Rab27b expression in these human pituitary adenoma tissues (Fig. 1M-P). A negative control was included. Nonspecificity of Rab27b was not expressed (Fig. 1Q-T).

The PRL and GH adenomas showed immunostaining of Rab3a (Fig. 1G, H), while full IHC analysis indicated that every section of functional adenoma tissue, including PRL and GH adenomas, was graded as positive for Rab3a expression (Table 2). In contrast, sections from the Null and Gn adenoma tissues were negative for Rab3a immunostaining (Fig. 1E, F), with the quantitation indicating that 43.5% (10/23) of Null adenoma samples and 25.0% (4/16) of Gn adenoma samples were Rab3a(−) (Table 2).

We next assessed the coexpression of Rab27b and Rab3a in adenoma cells by double immunofluorescence staining. The Rab3a(−) null samples (Fig. 2F, H) showed low Rab27b expression (Fig. 2A, C), as was the case for the Rab3a(−) Gn adenoma tissue sections (data not shown). In contrast, the Rab3a(+) PRL- and GH-producing functional adenoma tissues (Fig. 2I, J) also expressed Rab27b (Fig. 2D, F). The merged images clearly demonstrate coexpression at a cellular level, with both Rab3a and Rab27b visible in the cytoplasm of most pituitary adenoma cells (Fig. 2N, O). The areas of coexpression in the nonfunctional adenoma tissues (Fig. 2K-M) were less in extent than those in the functional adenomas (Fig. 2 F, H).

All functioning adenomas (PRL- and GH-producing tumors) showed positive staining for both Rab27b and Rab3a. In contrast, Rab27b(+) cells were largely absent from the NFPA tumors, including 69.6% (16/23) of Null adenomas and 37.5% (6/16) of Gn adenoma samples (Table 3).

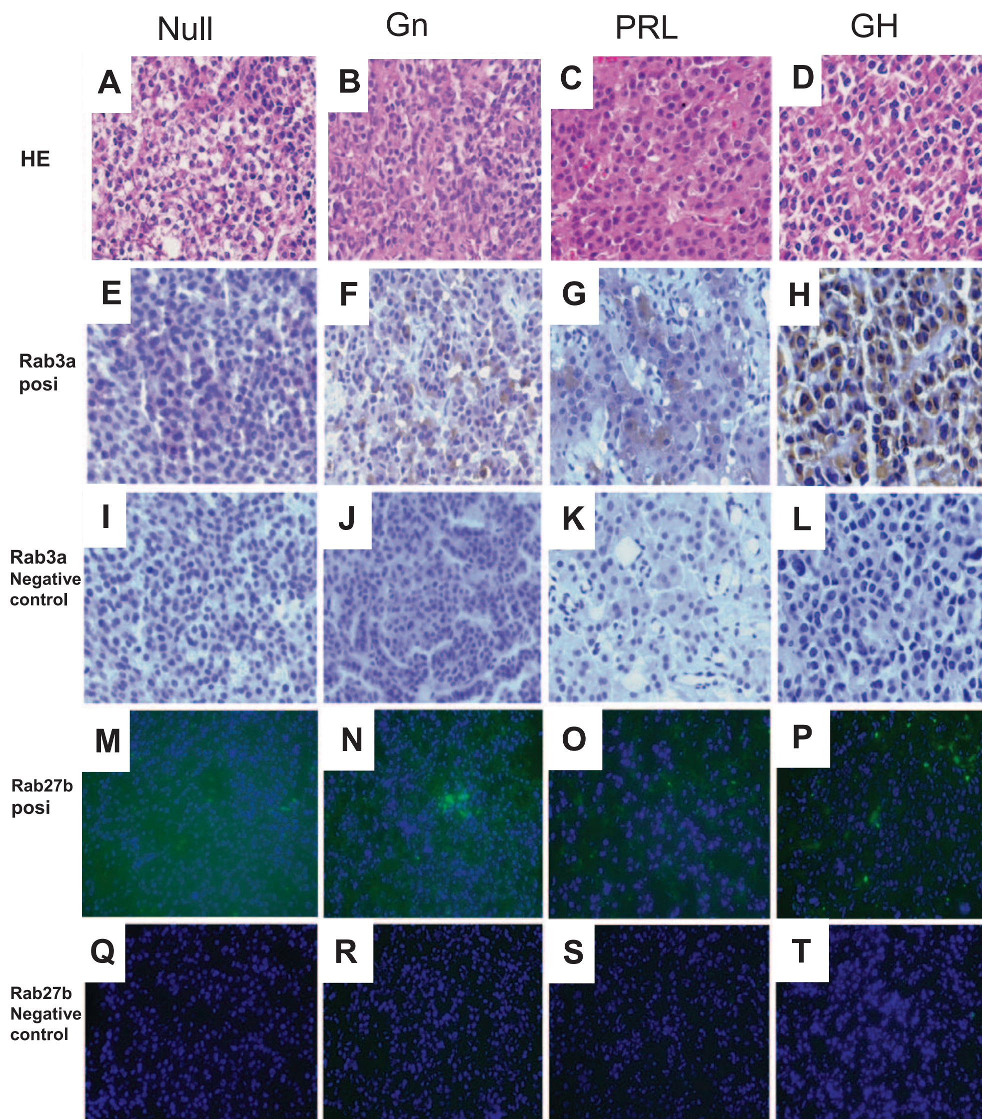

Fig. 1. Rab3a and Rab27b expression in human pituitary adenomas.
(A-D) Hematoxylin / eosin (HE)-stained sections of human pituitary adenomas, including null cell adenoma (Null) (A), gonadotropin adenoma (Gn) (B), prolactin secretion adenoma (PRL) (C), and growth hormone secretion adenoma. (GH) (D). Rab3a expression was detected in Null (E), Gn (F), PRL (G), and GH tissues (H). Rab3a was not expressed, Null (I), Gn (J), PRL (K), and GH tissues (L). Rab27b expression was detected in Null (M), Gn (N), PRL (O), and GH tissues (P). Rab27b were not expressed, Null (Q), Gn (R), PRL (S), and GH tissues (T). Magnification: ×400.

Discussion

Rab-family proteins are essential for all intracellular trafficking pathways. Rab proteins are small G proteins linked to biological membranes via lipid modifications. In neuroendo-

Table 2. Immunohistochemical expression of Rab3a

Rab3a expression				
Characteristics	n	Negative (%)	Positive (%)	*P* value
PRL adenoma	18	0	18 (100)	
GH adenoma	17	0	17 (100)	NS
Gonadotropin adenoma	16	4 (25)	12 (75)	0.072
Null cell adenoma	23	10 (43.5)	13 (56.5)	0.002

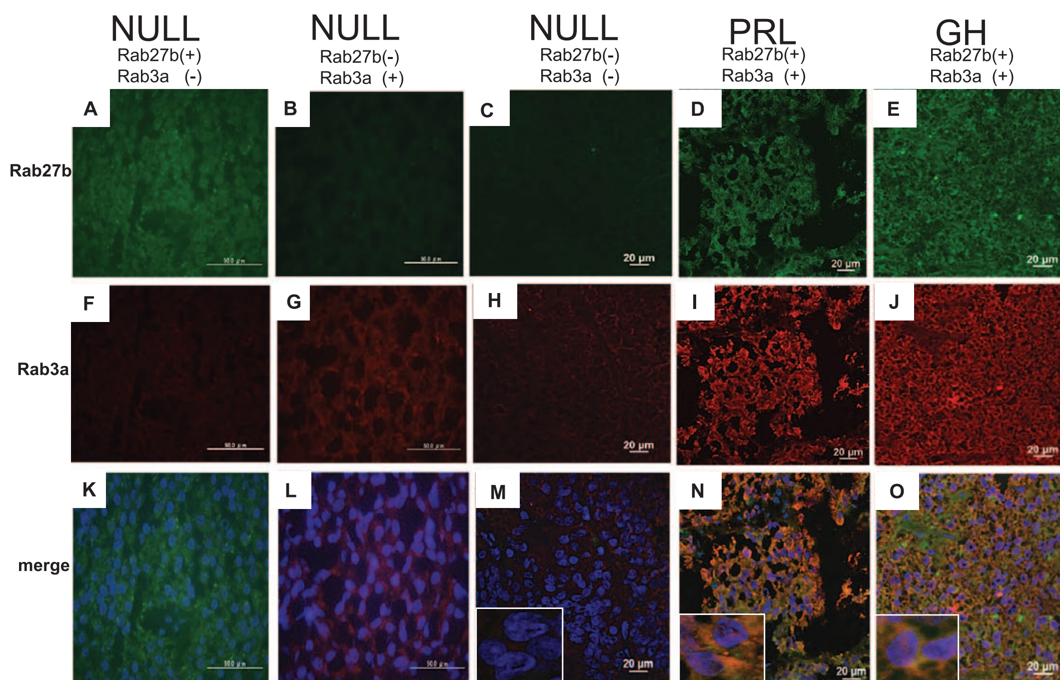

Fig. 2. Immunofluorescent staining of Rab3a and Rab27b.
Null Rab27b(+) Rab3a(−) (A), Rab27b(−) Rab3a(+) (B), Rab27b(−) Rab3a(−), (C), samples expressed lower levels of Rab27b than PRL (D) and GH (E). Rab3a expression was detected in Null Rab27b(−) Rab3a (+) (G), PRL (I), and GH (J). The merged images represent double staining of Null (K-M), PRL (N), and GH (O). Inset magnifications : ×600.

Table 3. Immunohistochemical expression of Rab27b

Rab27b expression				
Characteristics	n	Negative (%)	Positive (%)	*P* value
PRL adenoma	18	0	18 (100)	
GH adenoma	17	0	17 (100)	NS
Gonadotropin adenoma	16	6 (37.5)	10 (62.5)	0.006
Null cell adenoma	23	16 (69.6)	7 (30.4)	< 0.001

Table 4. Immunostaining of Rab27b and Rab3a in each pituitary adenoma

	Null cell adenoma (%)	Gonadotropin adenoma (%)	GH adenoma (%)	PRL adenoma (%)
Rab27b(−) Rab3a(−)	8 (34.7)	3 (18.7)	0	0
Rab27b(+) Rab3a(−)	2 (8.6)	1 (6.2)	0	0
Rab27b(−) Rab3a(+)	8 (34.7)	3 (18.7)	0	0
Rab27b(+) Rab3a(+)	5 (21.7)	9 (56.2)	17 (100)	18 (100)

crine cells, Rab proteins are recruited to the membranes of hormone-carrying vesicles and granules to help regulate both their trafficking and membrane fusion. Rab3a and Rab27b often cooperate in hormone-secretion pathways of neuroendocrine cells, and both have been associated with exocytotic vesicles[23,24].

In this study, all functioning pituitary adenoma tissues from our patient cohort (PRL and GH) were immunopositive for both Rab27b and Rab3a expression. In contrast, some of the nonfunctioning adenoma tissues (Null and Gn adenoma) expressed neither Rab27b nor Rab3a. Both Rab27b and Rab3a have been implicated as essential regulators of neurotransmitter and hormone secretion[25–28], and this study now suggests a potential clinical application of this basic knowledge about Rab proteins. Specifically, a Rab27b(−) Rab3a(−) pattern of expression might be a prognostic determiner of nonfunctioning adenoma cells, and IHC analysis for Rab27b and Rab3a could constitute part of the pathological testing.

Table 4 summarizes the coexpression of Rab3a and Rab27b in each pituitary adenoma. In total, 8 Null samples and 3 Gn adenoma tissues were negative for both Rab27b and Rab3a by IHC analysis 〔Rab27b(−) Rab3a(−)〕, suggesting that the vesicle trafficking system would be downregulated or defective in those coexpressing tumor cells. Contrary to our expectations, Rab27b(+) Rab3a(+) samples were also observed among the NFPA resections, suggesting the presence of other factors that could suppress hormone secretion such as inhibitors of protein synthesis, Rab inhibitors, or defective SNARE proteins. Gn adenoma cells have the capacity for hormone production, and the IHC experiments overall revealed various Rab expression patterns for the Gn adenoma sampled in this study, namely Rab27b(−) Rab3a(−), Rab27b(+) Rab3a(−), Rab27b(−) Rab3a(+), and Rab27b(+) Rab3a(+) (Table 4).

The two types of adenoma, Null and Gn are classified as NFPA, because these adenoma cells do not secrete hormones. However, these adenoma types differ in the degree of cellular differentiation. During the earliest stages of anterior pituitary development, the α-subunit of undifferentiated cells appears in Rathke's pouch, and specific factors act on Pit1 and Prop1 to induce the respective differentiation pathways to TSH-, PRL-, and GH-secreting cells[6]. We proposed therefore that when these transcription factors do not function, as in the Null adenomas, proper differentiation is halted and the various hormones are not made and not secreted[29]. In contrast, Gn adenoma can synthesize hormones, but

not secrete them. We propose therefore that Null adenoma is an undifferentiated pituitary tumor tissue, while Gn adenoma tissues have undergone some differentiation[30]. On the other hand, functioning pituitary adenomas (e.g., PRL and GH) represent highly differentiated pituitary tissues with proliferative capacity. Based on this study, we therefore concluded that the hormone synthesis system during the pituitary differentiation process was followed by development of the secretion mechanisms.

Null-cell adenoma cells do not synthesize hormone, but many differently sized vesicles (50–250 nm in diameter) can be seen inside these cells by electron microscopy. In addition, Gn adenomas cells contain many hormone-containing vesicles of a similar size (about 150 nm diameter)[31]. It is thought that nonfunctioning adenoma cells contain hormone-carrying vesicles, but do not secrete the vesicle contents[32]. The enrichment of vesicles observed in the nonfunctioning adenoma cells might therefore be attributed to defects in exocytosis that prevent vesicle fusion and release. Table 4 indicates that 64% of nonfunctioning adenoma samples (25 / 39 samples) in the present study were IHC-negative for Rab27b and / or Rab3a, supporting our initial hypothesis that such low expression might underlie the suspected exocytic trafficking defects based on reported functions for these Rabs in granule delivery at the cell membrane. Our results also confirmed that expression levels closely parallel the pathology of the NFPA. Indeed, localization of the Rab-immunopositive vesicles near the plasma membrane in some cells implied a problem with membrane fusion, but not with vesicle formation or delivery. All functioning pituitary adenomas studied here were IHC-positive for both Rab27b and Rab3a, corroborating reports that hormone secretion from pituitary endocrine cells requires the expression of these Rabs. In addition, the heterogeneous pattern of Rab27b / Rab3a expression in the various NFPA samples indicated that double IHC analysis of Rab27b and Rab3a could inform a more precise diagnosis of these types of pituitary adenoma.

The results of the present study showed expression of selected Rab proteins in all functioning hormone-secreting pituitary tumors, but at levels below the experimental detection limits in some NFPA. These findings suggested that the immunodetection of Rab proteins could supplement the pathological diagnosis of NFPA. In addition, there were both antibody-negative and antibody-positive samples analyzed in this group of patients, and it may be possible to further classify nonfunctioning adenomas based on hormone exocytosis and secretion. The number of cases analyzed herein was low, and further studies should include more Gn adenoma subjects for Rab3a immunostaining examination in particular. We would predict a significant difference in the number of Rab3a-negative cells between the Gn and functional adenomas. An interesting and unexpected result, given our hypothesis, was the presence of some Rab27b(+) Rab3a(+) samples among the NFPA patients. This was an apparent contradiction between the expected NFPA pathology and Rab-protein expression. Although further studies on these samples are needed, we would suggest that an inhibitor of exocytosis is acting in such cells to interrupt the Rab functions in membrane fusion and

hormone release.

In the future, we plan to establish the pathological classification of nonfunctioning tumors by analyzing the expression of Rab proteins in many patients with nonfunctioning pituitary tumors and develop a method to differentiate nonfunctioning adenoma cells at the molecular level.

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