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
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Association of PTP4A3 expression and tumour size in functioning pituitary adenoma: a descriptive study

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ABSTRACT

Background: PTP4A3 is a subclass of a protein tyrosine phosphatase super family and is expressed in a range of epithelial neoplasms. We evaluated PTP4A3 expression and its association with clinical-pathological parameters in different types of functioning pituitary adenomas.

Methods: A total of 34 functioning pituitary adenomas samples were evaluated in this observational study. PTP4A3 expression was examined by immunohistochemical staining, and, possible correlations between PTP4A3 and some clinical-pathological variables were investigated.

Results: PTP4A3 was expressed in 19 out of 34 tumors (55%), at the cytoplasmic level of tumoral cells. Moreover, there was significant association ($p=0.042$) between PTP4A3 expression and tumoral size.

Conclusions: PTP4A3 was expressed in more than half of the tumors analyzed, with there being a significant association with the tumoral size of functioning adenomas. This allows to speculate that PTP4A3 may regulate tumor growth, although further investigations are necessary to determine if this phosphatase can serve as a biomarker or used as a therapeutic target in pituitary macroadenomas.

BACKGROUND

The pituitary adenomas are one of the most frequent intracranial tumors, accounting for 10% of these and also 90% of intrasellar lesions with a prevalence of 1/1500, with approximately half of these tumors being functioning (46%–64%).^{1,2,3} Although pituitary adenomas are histologically benign, some may grow and become locally aggressive, resulting in a shortened lifespan.^{4,5} Different types of functioning pituitary adenomas are currently treated by medical therapies such as dopamine and somatostatin agonists, surgery or radiotherapy. However, these treatments are not entirely satisfactory and patients can fail to respond to traditional approaches.⁶

Protein tyrosine phosphatases (PTPs) are a large family of enzymes that catalyze the removal of phosphate groups attached to the tyrosine residues on their substrates, and are essential for regulating a myriad of cellular processes.⁷ In the tumoral pituitary gland, classical PTPs are considered to be cell growth regulators.⁸ Protein Tyrosine Phosphatase 4A3 (PTP4A3, also known as PRL-3) belongs to this group and has been implicated in the control of cell cycle, survival, angiogenesis, adhesion, cytoskeleton remodeling, epithelial-mesenchymal transition, motility and invasion.¹⁰ Moreover, PTP4A3 is a growth-associated protein in some tumoral types, with many studies having identified PTP4A3 to be a marker of tumor progression in different neoplasms.¹¹⁻¹⁴ Although an increased PTP4A3 expression, both at the mRNA and protein level, has been reported in several solid tumors, its expression in functioning pituitary adenomas has not yet been investigated.

Recently, a humanized antibody, PRL3-zumab, was shown to inhibit PTP4A3+ cancer cells *in vivo*, and thus this represents a feasible approach for anti-tumor immunotherapy.¹⁵ Taking into consideration this finding and that new therapeutic targets in functioning pituitary adenomas are required for improved endocrinological treatments, we performed an observational descriptive study of PTP4A3 expression in 34 functioning pituitary adenomas and investigated possible associations with some clinical-pathological parameters.

METHODS

Patients and Samples

A transsphenoidal surgery was performed on all sellar tumors, and the inclusion and exclusion criteria are shown in Table 1.

An observational, descriptive and retrospective study of normal pituitary glands (n:2; 30 min postmortem, aged 25-45, patients with no evidence of endocrine or histological abnormalities) and functioning pituitary adenomas of 34 adults (aged 20-64 years) was conducted at the Centro de Microscopía Electrónica-INICSA-Conicet.

Patients had undergone surgery at Hospital Córdoba, Sanatorio Allende or Clínica Reina Fabiola, Córdoba, Argentina between the years 2004-2015. These institutions performed the clinical diagnosis and reticulin staining, hormone analysis and Ki67 immunohistochemistry characterization, and also provided the samples in paraffin blocks.

The following data were collected: sex, age, tumoral size (macroadenoma/microadenoma), invasion, previous medical treatment, histopathology, hormone staining and Ki67 labeling index (Table 2).

Immunohistochemical staining

Sections 3µm thick were obtained from 34 functional pituitary adenomas and processed for immunohistochemistry. Briefly, the samples were deparaffinized in xylene and hydrated in alcohols, and the sections were heated in a microwave for 15 min in a citrate buffer (pH 6.0). Next, the sections were incubated first with hydrogen peroxide 0.5% solution in methanol to block endogenous peroxidase, and then with PTP4A3 rabbit polyclonal antibody (Abcam to anti-PTP4A3 ab82568, USA. 1/70) overnight at 4°C, and finally with biotinylated anti-rabbit antibody with ABC complex (Vector Laboratories, Burlingame, CA, USA). The reaction was detected using DAB as chromogen (Sigma, St. Louis, MO, USA). Positive controls for PTP4A3 were performed in human colon adenocarcinoma, with negative controls carried out by omitting the primary antibody incubation. The number of positively stained cells in the human adenomas was determined in 10 random fields at 400X. The total number of positive cells as a proportion of the number of cells in the field of vision was used to assign positive or negative values. If the percentage of positive cells was $\leq 10\%$, this was recorded as "0"; a percentage of between 11% and 25% was assigned "+"; a value of 26% -50% was recorded as "++"; a percentage of 51% -75% was assigned "+++" and finally, a value $> 76\%$ was recorded as "++++".

Western blot analysis

Protein extracts (50 µg) from 2 normal pituitary and 3 functioning pituitary adenomas (macro non-invasive corticotroph adenoma, macro invasive somatotroph

adenoma and macro invasive lactotroph) were separated in a polyacrylamide gel (Sigma–Aldrich), transferred to a nitrocellulose membrane (Amersham International), and the nonspecific binding was blocked with PBS-5% non-fat dried milk at RT. The membranes were then rinsed and incubated overnight with anti-PTP4A3 (Abcam to anti-PTP4A3 ab82568, USA) (1:166) or anti- β -Actin (1:4000; Sigma–Aldrich). The blots were incubated with HPRT-conjugated bovine anti-goat (1:2500; Santa Cruz Biotechnology), goat anti-mouse (1:2500, Jackson ImmunoResearch) or goat anti-rabbit secondary antibodies (1:5000, BioRad). The membranes were thoroughly rinsed in TBS 0.1% Tween 20, and the HPRT-coupled secondary antibody was revealed using enhanced chemiluminescence Western blotting detection reagents (GE Healthcare), with the emitted light captured on Hyperfilm (GE Healthcare).

Statistical analyses

Data were presented as mean (SD) for continuous data or for frequency and percentages (categorical data). We estimated the 95% confidence interval for the main quantitative variables. The T-test was used to compare PTP4A3 and the Ki67 labeling index between tumoral sizes (macroadenoma vs microadenoma). The Fisher exact test was used to assess an association between invasion and tumor size, a p value <0.05 was considered to be statistically significant for all the analyses. The statistical analysis was performed using the Stata 15.1 statistical package.¹⁶

RESULTS

PTP4A3 expression in functional pituitary adenomas

Pituitary adenomas were characterized by the H/E stain, immunohistochemistry hormone determination and transmission electron microscopy (figure 1A-D). In this cohort, 59% of patients were female, with the mean (SD) age being 39 (12.96) years.

The clinico-pathological findings revealed that there were 29% lactotroph cell adenomas, 20% somatotroph adenomas, 11% mammosomatotropic adenomas, 35% corticotroph cell adenomas and 3% tirotroph cell adenomas (figure 1). Of the above, 56% were macroadenomas and 44% microadenomas. As 4 of the lactotroph cell adenomas were resistant to previous medical treatment (dopamine agonist), these were treated surgically.

The mean (SD) of the Ki67 index was 2.68 (0.39); CI 95% [1.88-3.48]. Sixty-eight percent of the adenomas revealed a Ki67 index equal to or less than 3%, with the

Ki67 index being independent of tumor size ($p > 0.05$). Unfortunately, the presence or absence of invasion could only be determined in 10 out of 34 functional pituitary adenomas.

The PTP4A3 expression in 34 functioning pituitary adenomas was determined by immunohistochemistry, with the mean (SD) of PTP4A3 being 23.02857 (4.72); CI 95% [13.42- 32.62]. As shown in Figure 1-F, PRL-3 protein was mainly localized in the cytoplasm, which sometimes occurred as granulated loci in the strongly positive samples. According to the criteria, the PTP4A3 positive expression rate was 64% in the adenoma group (Table 3). The semi-quantitative phosphatase analysis showed that there was a higher expression of PTP4A3 in proliferative pituitary lesions than in normal glands (figure 1-I). However, there was only a significant and greater difference found for PTP4A3 between pituitary macro adenomas and micro adenomas ($p=0.042$) (Table 4). Finally, for the adenoma pituitary lineage-cells, the PTP4A3 expression was similar in the PRL-STH-TSH (54%) lineage and ACTH lineage (58%).

DISCUSSION

Functioning pituitary tumors are benign proliferations with different biological characteristics and behaviors, especially with regard to tumor size and invasion.⁴ Similar to other series reported, in our cohort 59% of patients were female, but the mean age was about 39 years lower than for other studies.³ Women were predominant and at an earlier age and most markedly for corticotroph tumors, as was previously reported.³

We conducted an observational descriptive study to evaluate PTP4A3 expression in the normal pituitary gland and in a functioning adenoma cohort. This phosphatase is in normal tissue mainly expressed in the heart, skeletal muscle, and to a lesser extent in the prostate.⁹ PTP4A3 also shows low basal levels in the human normal pituitary gland in agreement with that the fact that phosphatase is expressed at low levels in other normal human epithelial tissue.⁹ However, this PTP has largely unknown physiological cellular functions, although it has been associated with terminal cell differentiation as well as appearing to be important in ensuring cell cycle progression by facilitating G1/S transition.^{9,17}

In our investigation, 19 out of 34 functioning pituitary adenomas exhibited detectable immunolabeling of PTP4A3, with 26% to 50% of the immunopositive cells occurring in more than half of PTP4A3 positive adenomas. One important point was that 63.63% of somatotroph adenomas, 58.33% of corticotroph adenomas and 50 % of

lactotroph adenomas were PTP4A3 positive tumors. To date, few pituitary investigations have reported PTPs' expression. It has been proposed that the 'classical PTPs', namely SHP-1, SHP-2, and DEP-1/PTPn, play a pivotal role in SRIF/SRL-mediated control of cell growth, and seem to be activated by ligand-binding to all the different SST subtypes. Cells transfected with the individual somatostatin receptor (SSTR) subtypes have demonstrated that all the 5 SSTRs are able to induce PTP activity.^{8,18} However, we report for first time a PTP4A3 increased expression in a group of different cell lineage pituitary adenomas. Although no substrate has yet been clearly identified for the PTPs, a few have been suggested for PTP4A3, such as the PI3K-Akt pathway, although the direct mechanism involved still remains unclear.¹⁹

Monsalves et. al. suggested that the pituitary adenoma growth rate is influenced by various patient- and tumor-specific characteristics, such as age, sex, specific subtype, hormonal activity and immunohistochemical profile, including the mindbomb homolog 1 labeling index status.²⁰ Here, we demonstrated that there was significant difference between PTP4A3 expression and pituitary adenoma size, thereby revealing a possible new molecule that may regulate tumor growth, at least in some adenomas. We also consider that our findings have identified a new concept in pituitary pathology, in which a group of PTP4A3 positive macroadenomas could be a potential therapy target for new treatments to reduce the tumoral size, and may be also utilized as a postoperative adjuvant therapy to prevent recurrence.¹⁵ Although we cannot extrapolate the results to fit the entire population (inclusive bias), a further multicenter study with a large sample size should be conducted, to determine if PTP4A3 immunostaining could also be used to identify potential patients as a PRL3-zumab target.

Take home messages

- PTP4A3 is expressed in the normal pituitary gland.
- Overexpression of PTP4A3 was observed in a series of 19 patients with pituitary adenomas, with phosphatase expression occurring in the PRL-GH-TSH lineage and ACTH-lineage cells.
- There was a significant difference between PTP4A3 expression in pituitary macro adenomas vs. micro adenomas.

Contributors All authors contributed to the design of the study, writing, or critical, review of the manuscript, analysis and interpretation of data. All agreed to submission of the manuscript.

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Competing interests None declared.

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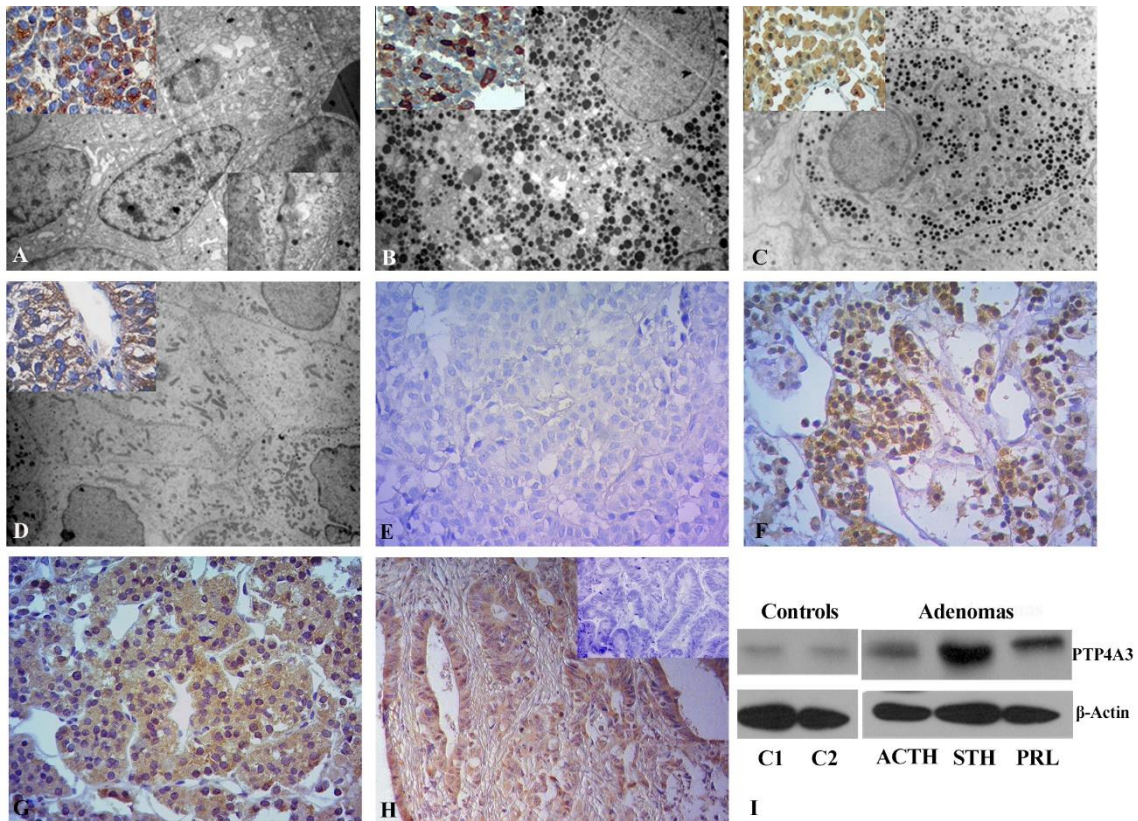


Figure 1 Ultrastructure and hormone immunolabeling in lactotroph (A), somatotroph (B), corticotroph (C) and tirotroph (D) adenomas. E-Negative control of PTP4A3 immunolabeling in pituitary adenomas; F: PTP4A3 expression, mainly localized in the cytoplasm (x400, magnification). G: +++ PTP4A3 expression, H: Positive PTP4A3 control in colon cancer sample. Inset: negative control omitting primary antibody. I: PTP4A3 semiquantitative analysis in normal gland and different pituitary adenomas.

Table 1 Patient inclusion and exclusion criteria

Patient inclusion criteria
- Female and male patients older than 18 years old
- Functioning pituitary adenomas
- Availability of clinical data (previous medical treatment medical, tumoral size, tumoral invasion)
- Availability of immunolabelling report of adenohipofyseal hormones and Ki67
Patient exclusion criteria
- Sellar non-pituitary adenomas (neuronal and paraneuronal tumors; mesenchymal tumors; germ cell tumors; hematological tumors; secondary tumors)
- Non-functioning pituitary adenomas

Table 2 Clinical variables and pathological parameters

Case	Gender	Age	Size	Invasion	Previous medical treatment	Histopathology	Hormone IHC	KI 67 %
1	M	59	M	Yes	Naive to treatment	Sparsely granulated lactotroph adenoma	PRL	8
2	M	51	M	Yes	Naive to treatment	Sparsely granulated lactotroph adenoma	PRL	1
3	F	30	M	NA	Naive to treatment	Sparsely granulated lactotroph adenoma	PRL	1
4	M	27	M	NA	Naive to treatment	Sparsely granulated lactotroph adenoma	PRL	10
5	M	35	M	Yes	Naive to treatment	Sparsely granulated lactotroph adenoma	PRL	5
6	M	60	M	NA	Naive to treatment	Sparsely granulated lactotroph adenoma	PRL	5
7	F	20	m	NA	Resistant	Sparsely granulated lactotroph adenoma	PRL	1
8	F	38	m	NA	Resistant	Sparsely granulated lactotroph adenoma	PRL	1
9	F	31	m	NA	Resistant	Sparsely granulated lactotroph adenoma	PRL	1
10	F	26	m	NA	Resistant	Sparsely granulated lactotroph adenoma	PRL	1
11	M	57	M	NA	Naive to treatment	Densely granulated somatotroph adenoma	STH	2
12	F	33	M	Yes	Naive to treatment	Densely granulated somatotroph adenoma	STH	1
13	F	64	M	NA	Naive to treatment	Sparsely granulated somatotroph adenoma	STH	0
14	F	49	M	NA	Naive to treatment	Sparsely granulated somatotroph adenoma	STH	1
15	M	29	M	NA	Naive to treatment	Sparsely granulated somatotroph adenoma	STH	3
16	F	58	m	NA	Naive to treatment	Densely granulated somatotroph adenoma	STH	3
17	M	37	m	NA	Naive to treatment	Sparsely granulated somatotroph adenoma	STH	2
18	F	35	M	NA	Naive to treatment	Mammosomatotroph adenoma	STH/PRL	4
19	M	28	M	NA	Naive to treatment	Mammosomatotroph adenoma	SHT/PRL	2
20	M	53	m	NA	Naive to treatment	Mammosomatotroph adenoma	SHT/PRL	5
21	F	41	m	NA	Naive to treatment	Mammosomatotroph adenoma	STH/PRL	1
22	M	32	m	No	Naive to treatment	Thyrotroph adenoma	TSH	5
23	M	35	M	Yes	Naive to treatment	Densely granulated corticotroph adenoma	ACTH	1
24	F	32	M	NA	Naive to treatment	Densely granulated corticotroph adenoma	ACTH	5
25	F	62	M	Yes	Naive to treatment	Densely granulated corticotroph adenoma	ACTH	2
26	F	51	M	NA	Naive to treatment	Densely granulated corticotroph adenoma	ACTH	3
27	F	31	M	NA	Naive to treatment	Densely granulated corticotroph adenoma	ACTH	2
28	F	41	M	NA	Naive to treatment	Densely granulated corticotroph adenoma	ACTH	2

29	F	55	m	No	Naive to treatment	Densely granulated corticotroph adenoma	ACTH	0
30	F	23	m	No	Naive to treatment	Densely granulated corticotroph adenoma	ACTH	3
31	M	30	m	No	Naive to treatment	Densely granulated corticotroph adenoma	ACTH	3
32	F	21	m	NA	Naive to treatment	Densely granulated corticotroph adenoma	ACTH	1
33	F	25	m	NA	Naive to treatment	Densely granulated corticotroph adenoma	ACTH	7
34	F	38	m	NA	Naive to treatment	Densely granulated corticotroph adenoma	ACTH	1

Gender: M: male, F: female; Size: M: macroadenoma, m: microadenoma; Invasion: NA: not available; PRL: prolactin; STH: somatotroph hormone; TSH: tirotroph hormone; ACTH: adrenocorticotroph hormone.

Table 3 PTP4A3 expression in pituitary adenomas

Adenoma	<i>n</i>	Neg	1+	2+	3+	4+
PRL	10	5	2	2	0	1
GH	11	4	3	4	0	1
TSH	1	1	0	0	0	0
ACTH	12	5	0	5	0	2

Table 4 Relationship of PTP4A3 positive expression and pituitary adenomas clinico-pathological features

Clinical Data	<i>n</i>	PRL-3 positive expression	
		Positive (Cases)	Percentage (%)
Age			
>55	9	3	33,33
<55	25	16	64
Gender			
men	13	8	61,53
women	21	11	52,38
Adenoma			
PRL	10	5	50
GH	11	7	63,63
TSH	1	0	0
ACTH	12	7	58,33
Tumoral Size			
Macro	19	13	68,42
Micro	15	6	40

