Differential Expression of Stem Cell Markers in Human Adamantinomatous Craniopharyngioma and Pituitary Adenoma

Claudia Veiga Chang^a Ricardo Vieira Araujo^{a,g} Cinthya Santos Cirqueira^b
Carolina Maria Gomes Cani^a Hamilton Matushita^c
Valter Angelo Sperling Cescato^d Maria Candida Barisson Villares Fragoso^a
Marcello Delano Bronstein^e Maria Claudia Nogueira Zerbini^f
Berenice Bilharinho Mendonca^a Luciani Renata Carvalho^a

^aLaboratório de Hormônios e Genética Molecular – LIM/42, Divisão de Endocrinologia, FMUSP, ^bInstituto Adolfo Lutz, Centro de Patologia, Núcleo de Anatomia Patológica, ^cDivisão de Neurocirurgia do Hospital das Clínicas, FMUSP, ^dDivisão de Neurocirurgia Funcional do Instituto de Psiquiatria do Hospital das Clínicas, FMUSP, ^eServiço de Neuroendocrinologia, Divisão de Neurocirurgia Funcional do Instituto de Psiquiatria do Hospital das Clínicas da FMUSP, ^fDepartamento de Patologia, Faculdade de Medicina da Universidade de São Paulo, and ^gMinistério da Ciência, Tecnologia & Inovação, Secretaria de Políticas e Programas de Pesquisa e Desenvolvimento, Brasília, Brazil

Key Words

Stem cell markers · Tumour · Pituitary adenoma · Craniopharyngioma

Abstract

Background/Aims: Although craniopharyngioma (CP) is histologically benign, it is a pituitary tumour that grows rapidly and often recurs. Adamantinomatous CP (ACP) was associated with an activating mutation in β -catenin, and it has been postulated that pituitary stem cells might play a role in oncogenesis in human ACP. Stem cells have also been identified in pituitary adenoma. Our aim was to characterize the expression pattern of *ABCG2*, *CD44*, *DLL4*, *NANOG*, *NOTCH2*, *POU5F1/OCT4*, *SOX2*, and *SOX9* stem cell markers in human ACP and pituitary adenoma. **Methods and Results:** We studied 33 patients (9 ACP and 24 adenoma) using real-time quantitative PCR (RT-qPCR) and immunohistochemistry.

SOX9 was up-regulated in ACP, exhibiting positive immunostaining in the epithelium and stroma, with the highest expression in patients with recurrence. *CD44* was overexpressed in ACP as confirmed by immunohistochemistry. *SOX2* did not significantly differ among the tumour types. The RT-qPCR array showed an increased expression of *MKI67*, *OCT4/POU5F1*, and *DLL4* in all tumours. *NANOG* was decreased in ACP. *ABCG2* was down-regulated in most of the tumours. *NOTCH2* was significantly decreased in the adenomas. **Conclusion:** Our results confirm the presence of stem cell markers in human pituitary tumours as well as the different expression patterns of ACP and adenoma. These findings suggest that ACP may originate from a more undifferentiated cell cluster. Additionally, *SOX9* immunodetection in the stroma and the highest expression levels related to the relapse of patients suggest a contribution to the aggressive behaviour and high recurrence of this tumour type.

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Introduction

The pituitary gland is an important endocrine organ that controls basic physiological processes, such as growth, fertility, metabolism, and stress response. The pituitary hormones GH, LH/FSH, PRL, ACTH, and TSH act on organs and endocrine glands to moderate physiological processes throughout life. Puberty and pregnancy in humans are physiological conditions that necessitate an increase in the size of the pituitary gland. Under such conditions in mice, pituitary plasticity depends on the asymmetric proliferation of adult stem cells followed by their terminal differentiation into hormone-producing cells [1–3]. Previous studies have demonstrated the presence of stem cell markers (such as SOX2 and SOX9) in normal adult pituitary tissues from humans and animals [4].

Stem cells have also been isolated from human pituitary adenoma. They have been demonstrated to form pituitary spheres and to secrete hormones in culture [5, 6]. Pituitary adenomas are benign tumours, but their hormone production and associated growth disorders are related to increased morbidity [7].

The presence of the SOX2 and SOX9 stem cell markers has also been demonstrated in the pituitary tissues of the transgenic mouse model of craniopharyngioma (CP) and in human adamantinomatous craniopharyngioma (ACP) samples [8, 9].

CPs are classified into two clinicopathological types: ACP, which has a bimodal incidence pattern (the majority of patients are under 15 years of age, with a second peak occurring in adults between 50 and 74 years of age), and papillary CP (PCP), which is less frequent and occurs during adulthood [10, 11]. ACP and PCP are histologically different. Classic features of ACP are defined by the presence of wet keratin nodules, calcification, and/or stellate reticulum surrounded by peripheral palisaded epithelium, and PCP is defined by the absence of wet keratin nodules, calcification, and/or stellate reticulum and the presence of squamous epithelium with fibrovascular cores. Complete resection is nearly impossible due to the invasion of adjacent tissues. In some cases, radiotherapy is necessary as an adjuvant treatment, but it is associated with many adverse effects and high recurrence rates [12].

ACP, but not PCP, has been associated with a β -catenin mutation in exon 3 that prevents its degradation and allows it to enter into the nucleus to promote proliferation and growth. Despite the reported association of β -catenin mutations with ACP, some patients do not carry a mutation in this gene [13, 14].

All of the recently acquired knowledge of stem cells in relation to CP physiopathology belongs to ACP and not PCP. *CD44* is another stem cell marker expressed in ACP. It is a transmembrane glycoprotein involved in cell migration, adhesion, and maintenance of stem cell characteristics [15], and it has been associated with aggressive behaviour, dissemination, and poor prognosis in a variety of human tumours.

The stem cell markers SOX2 [16], *POU1F5/OCT4* [16], SOX9 [16, 17], and *CD44* [18] have previously been described in association with ACP. In this study, we aimed to increase current knowledge regarding the expression patterns of pituitary stem cell markers in ACP and pituitary adenomas, including the markers *DLL4*, *NANOG*, *NOTCH2*, and *POU1F5/OCT4*, to better understand the tumour development and recurrence process.

Methods

Ethics Statement

The Human Research Ethics Committee of the University of São Paulo Medical School approved the protocol, and all participants provided written informed consent. Clinical investigations were conducted according to the principles of the Declaration of Helsinki.

Patient and Tumour Characterizations

Thirty-three tumour samples were obtained at the time of surgery at the Hospital das Clínicas of the University of São Paulo from 1993 to 2010. The surgical specimens were immediately frozen in liquid nitrogen and stored at -80°C . The remaining fragments were subjected to routine histological and immunohistochemical analyses.

Patients

Thirty-three patients were selected, and their clinical features are summarized as follows: 10 patients (7 females and 3 males) had Cushing's disease, including 4 due to microadenoma, 5 due to macroadenoma, and 1 due to Nelson's syndrome. At the time of surgery, the age of the patients was between 15 and 35 years. Two patients were taking cabergoline only, 2 were taking ketoconazole only, 1 was taking both cabergoline and ketoconazole, and another was taking both cabergoline and octreotide (table 1). Eight patients (4 females and 4 males) were diagnosed with acromegaly due to macroadenoma. These patients underwent surgery at between 33 and 71 years of age. Six patients (3 females and 3 males) were diagnosed with non-functional macroadenoma and, at the time of surgery, they were between 47 and 66 years of age. Nine patients (7 females and 3 males) had ACP, and they underwent surgery at between 3 and 21 years of age. None of these patients were taking medication (table 1).

Real-Time Quantitative PCR

After surgical tumour resection, total RNA was extracted using Trizol reagent (Life Technologies, Carlsbad, Calif., USA) accord-

Table 1. Clinical and pathological characteristics of patients with different tumours

Pa-tient	Clinical features	Sex	Age at surgery, years	Tumour size, cm	Immunohistochemistry ^a	Medication at surgery
1	Cushing's disease	F	35	1.8	ACTH 3+	–
2	Cushing's disease	M	16	0.9	ACTH 2+	–
3	Cushing's disease	M	18	1.0	ACTH 3+	–
4	Cushing's disease	F	27	2.0	ACTH 3+	Cabergoline and ketoconazole
5	Cushing's disease	F	24	1.0	ACTH 3+	Ketoconazole
6	Cushing's disease	F	34	0.6	ACTH 2+	–
7	Cushing's disease	F	34	1.0	ACTH 2+	–
8	Cushing's disease	M	15	0.4	ACTH 2+	Ketoconazole
9	Cushing's disease	F	19	0.6	ACTH 2+	Cabergoline and octreotide
10	Nelson's syndrome	F	33	>1.0	ACTH 2+	–
11	Acromegaly	M	71	1.7	GH 3+, FSH 1+	–
12	Acromegaly	M	45	6.4	GH 3+, PRL 3+	–
13	Acromegaly	M	33	1.2	GH 3+, LH 2+, FSH 2+	–
14	Acromegaly	M	40	5.9	GH 2+, PRL 1+	Cabergoline
15	Acromegaly	F	37	2.2	GH 3+, LH 1+, TSH 1+, PRL 3+	–
16	Acromegaly	F	59	1.1	GH 3+	–
17	Acromegaly	F	53	2.2	GH 3+, FSH 1+, TSH 1+	–
18	Acromegaly	F	54	7.0	GH 3+, FSH 1+	–
19	Non-functioning adenoma	M	51	2.5	TSH 1+	–
20	Non-functioning adenoma	F	50	2.6	TSH 2+	–
21	Non-functioning adenoma	M	56	3.4	LH 1+, FSH 1+	–
22	Non-functioning adenoma	F	66	3.3	–	–
23	Non-functioning adenoma	F	47	2.4	–	–
24	Non-functioning adenoma	M	62	6.0	GH 1+, ACTH 1+	Cabergoline
25	CP	M	21	2.0	NP	–
26	CP	M	9	3.5	NP	–
27	CP	F	17	2.5	NP	–
28	CP	F	3	5	NP	–
29	CP	F	11	2.0	NP	–
30	CP	F	9	3.0	NP	–
31	CP	F	7	2.5	NP	–
32	CP	F	10	4	NP	–
33	CP	F	14	2.7	NP	–

Nuclear immunostaining of each sample was evaluated with a semi-quantitative scale as follows: – = no immunostaining, 1+ = 1–10% of immunoreactive cells, 2+ = 11–50% of immunoreactive cells, and 3+ = >50% of immunoreactive cells. NP = Not performed.

^a Immunostaining using anti-pituitary hormone antibodies.

ing to the manufacturer's protocol. Total RNA pooled from 88 human pituitary glands (Clontech, Mountain View, Calif., USA) was used as a control. We used 5 µg total RNA from each tumour sample or human pituitary gland total RNA (Clontech, Mountain View, Calif., USA) for cDNA synthesis employing an RT² First Strand Kit (SABiosciences; Qiagen, Valencia, Calif., USA), including a genomic DNA decontamination step. Real-time quantitative PCR (RT-qPCR) was performed with the help of a custom RT² ProfilerTM PCR Array – CAPH09297A (SABiosciences) with SYBR Green I as a DNA-binding dye. The genes represented in the customized array were selected based on previously published microarray results obtained using normal adult mouse pituitary stem/progenitor cells [19] and included *ABCG2*, *CD44*, *DLL4*, *NANOG*,

NOTCH2, *POU1F5/OCT4*, *SOX2*, and *SOX9*. The RT-qPCR array also included the cellular proliferation marker *MKI67* (table 2). First-strand cDNA was subjected to PCR under the following conditions: 95°C for 10 min and 40 amplification cycles at 95°C for 15 s and 60°C for 1 min followed by melting curve analysis. Relative expression levels compared to normal pituitary gland expression were calculated with the $2^{-\Delta\Delta CT}$ method using the geometric means of the Ct values of eight housekeeping genes (table 2). Quantitative measurements were performed in quadruplicate. All samples fulfilled the following criteria for adequate RNA quality: the use of a reverse transcriptase cDNA control and PCR-positive control, the absence of genomic DNA, and housekeeping gene expression corresponding to a low Ct value. All RNA samples had a

Table 2. Genes studied in the RT-qPCR array

Gene	GenBank number	Predicted function
Stem/progenitor cell		
ABCG2	NM_004827	Cellular membrane transporter protein
CD44	NM_001001391	Cell adhesion molecule
DLL4	NM_005618	Delta family NOTCH ligand
NANOG	NM_024865	Transcription factor
NOTCH2	NM_024408	Cellular membrane receptor
POU5F5/OCT4	NM_203289	Transcription factor, POU homeodomain family member
SOX2	NM_003106	Transcription factor, SRY-related HMG-box family member
SOX9	NM_000346	Transcription factor, SRY-related HMG-box family member
Proliferation marker		
MKI67	NM_002417	Nuclear protein
Housekeeping genes		
PPIA	NM_021130	Peptidylprolyl isomerase A
PGK1	NM_000291	Phosphoglycerate kinase 1
TBP	NM_003194	TATA box binding protein, transcription factor
B2M	NM_004048	β_2 -Microglobulin
HPRT1	NM_000194	Hypoxanthine phosphoribosyltransferase 1
RPL13A	NM_012423	Ribosomal protein L13a
GAPDH	NM_002046	Glyceraldehyde-3-phosphate dehydrogenase
ACTB	NM_001101	Actin
Array controls		
HGDC	SA_00105 ^a	Human genomic DNA contamination
RTC	SA_00104 ^a	Reverse transcription control
PPC	SA_00103 ^a	Positive PCR control

^a SABiosciences; Qiagen RefSeq#.

Table 3. Differential gene expression obtained by RT-qPCR array

Genes	ACTH_PA (n = 10)	GH_PA (n = 8)	NF_PA (n = 6)	ACP (n = 9)
Stem/progenitor cell markers				
ABCG2	-1.51 (0.89)	-2.11 (1.12)	-1.82 (1.16)	-2.65 (1.32)
CD44	0.81 (1.23)	0.5 (1.48)	-1.01 (1.65)	6.62 (0.21)
DLL4	3.63 (0.59)	2.27 (1.00)	2.75 (0.44)	1.99 (2.13)
NANOG	0.18 (0.48)	-0.58 (0.49)	-0.003 (0.48)	-2.33 (0.90)
NOTCH2	-2.92 (2.46)	-4.06 (0.87)	-3.87 (0.66)	0.49 (0.59)
POU5F1/OCT4	3.76 (0.24)	4.34 (0.64)	4.01 (0.45)	2.76 (0.81)
SOX2	-0.59 (1.63)	-0.34 (1.67)	-0.73 (0.82)	1.94 (3.20)
SOX9	-0.64 (2.97)	-2.34 (1.39)	-4.42 (1.39)	3.05 (1.31)
Cellular proliferation				
MKI67	4.03 (1.15)	1.47 (1.11)	2.26 (0.54)	3.91 (0.77)

Values are presented as means of relative expressions calculated by the $\Delta\Delta^{CT}$ method represented as log of the base 2, and the numbers in parentheses represent the standard deviation. The normal range was considered to be between -1.32 and +1.32. ACTH_PA = ACTH-producing adenomas; GH_PA = GH-producing adenomas; NF_PA = non-functioning adenomas.

PCR-positive control Ct value that was within the adequate range of quality (20 ± 2) indicated by the manufacturer. Because the relative quantification error of this method was a 2.5-fold change according to the manufacturer (Applied Biosystems, Foster City, Calif., USA), this value was selected as the cut-off value. We pre-

sented the fold changes as $\log_2$ ratios. As the $\log_2$ ratio of the cut-off value is 1.32, genes with fold changes of between -1.32 and +1.32 were considered to be within the normal range.

PCR was performed with the help of an ABI PRISM 7000 Sequence Detection System (Applied Biosystems).

Fig. 1. Differential gene expression of stem/progenitor cells and proliferation markers in pituitary adenomas and CPs. Normalized expression levels, as determined by RT-qPCR array, are presented as the scatter plot mean and standard deviation (SD). The values on the y-axis represent the fold change log 2. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. C = Corticotropinoma; S = somatotropinoma; NF = non-functioning adenoma; ACP = adamantinomatous craniopharyngioma.

Immunohistochemistry

Nine paraffin blocks were available for immunostaining, including 5/10 corticotropinomas, 1/8 somatotropinomas, 1/6 non-functioning adenomas, and 2/9 ACPs. Each paraffin block was sliced into 3- μ m-thick sections using an automatic Leica RM2255 microtome (Leica Biosystems, Nussloch, Germany). The sections were stretched in a water bath at 50°C and completely dried in a FANEM Orion model 515 incubator (FANEM, São Paulo, Brazil) at 60°C. Each section was subjected to haematoxylin-eosin staining for histological analysis. Slides were deparaffinized with xylene, hydrated in ethanol, washed in phosphate-buffered saline

(0.01 M/pH 7.4) and blocked in methanol and hydrogen peroxide. Epitope exposure was carried out by placing the slide in boiling 10 mM citric acid (pH 6) or 100 mM EDTA (pH 9) followed by the blocking of non-specific proteins. The following primary antibodies against hormones were added: ACTH, mouse monoclonal [02A3] (M3501, DAKO): 1:10,000; GH, rabbit polyclonal (208A, Cell Marque): 1:2,000; FSH, mouse monoclonal [C10] (M3504, DAKO): 1:20,000; LH, rabbit polyclonal (209A, Cell Marque): 1:50; CD44, rabbit monoclonal [EPR1013Y] (ab51037, Abcam, San Francisco, Calif., USA): 1:1,000, pH 6.0; SOX9, mouse monoclonal [3CD10] (ab76997, Abcam): 1:20,000, pH 9.0, and SOX2,

Fig. 2. SOX9 differential expression, as determined by immunohistochemistry, in ACPs and adenomas. **a** CP. HE. $\times 200$. **b** CP. H-score = 20. $\times 200$ for both epithelia and stroma (arrow). **c** CP negative. $\times 200$. **d** Corticotropinoma. H-score = 8. $\times 400$. **e** Corticotropinoma. H-score = 20. $\times 400$. **f** Corticotropinoma negative. $\times 400$. **g** Tonsil positive control $\times 400$. SOX9, 1:20,000.

rabbit polyclonal (ab97959, Abcam): 1:400, pH 6.0. After the addition of the antibodies, the samples were left at 4°C overnight. Subsequently, the sections were incubated with polymer-based secondary antibodies labeled with horseradish peroxidase according to the manufacturer's instructions (Novolink Polymer Detection Systems; Leica Biosystems, Newcastle, UK). The reaction was then visualized with 3,3'-diaminobenzidine tetrahydrochloride (Sigma D5637, Sigma-Aldrich, St. Louis, Mo., USA). Negative control samples were performed without the primary antibody and adding the secondary antibody as described previously.

The immunohistochemical expression of SOX9 was scored semi-quantitatively in the neoplastic epithelial and stromal tissues separately as well as together in both tissues. Positive staining was classified as follows: 0 = no stained cells; 1 = up to 10% stained cells; 2 = from 11 to 20% stained cells; 3 = from 21 to 30% stained cells, and up to 10 = from 91 to 100% stained cells. SOX9 staining intensity was graded as follows: 0 = negative; 1 = weak, or 2 = strong. The expression score was calculated by multiplying the proportion of stained cells by the grade of SOX9 staining intensity. Thus, the final expression score ranged from 0 to 20, with 0 indicating no expression and 20 indicating the highest expression [modified from 20].

The tissue samples were fully examined under a multi-head microscope by 3 different observers, who reached a consensus to determine the final scores.

Statistical Analysis

All relative expression data (expressed as $\log_2$ values) were analysed by one-way analysis of variance (ANOVA). Tukey's correction was applied to assess significant differences between the tumour groups, and differences were considered statistically significant at a p value of <5% ($p < 0.05$). Analyses were performed using Sigma Stat (Version 3.5, Germany).

Results

The RT-qPCR array results for the pituitary adenoma and ACP patients are displayed in table 3. All of the ACP patients had been genotyped for exon 3 β -catenin mutations, and 5 of them were found to carry a mutation. These data have previously been published [13].

Fig. 3. CD44 differential expression as determined by immunohistochemistry. **a** Negative expression in adenoma, with lymphocyte immunostaining used as an internal control (arrow). $\times 200$. Highly positive immunostaining in ACP: $\times 200$ (**b**), $\times 200$ (**c**), and $\times 400$ (**d**).

Fig. 4. SOX2 differential expression as determined by immunohistochemistry in ACPs and adenomas. **a** CP. H-score = 20. $\times 200$. **b** CP. H-score = 20. $\times 200$. **c** Corticotropinoma. H-score = 5. $\times 200$. **d** Corticotropinoma. H-score = 1. $\times 200$. SOX2, 1:400.

RT-qPCR showed increased SOX9 expression in the ACPs compared to the pituitary adenomas ($p < 0.05$; table 3; fig. 1). As shown in figure 2, SOX9 nuclear immunostaining was observed in the palisaded basal cell layer in the epithelium and in stellate cells in the stroma under the epithelium (fig. 2).

In addition, the stem cell marker *CD44* was highly expressed in the ACPs, with an average 6.62-fold increase in expression compared to normal pituitary ($p < 0.001$; table 3; fig. 1). *CD44* membrane immunostaining was also observed in the palisaded basal cell layer in the epithelium (fig. 3). SOX2 expression was detected in both the

ACPs and adenomas, but no significant difference was found between the two tumour types (table 3; fig. 1, 4). *NANOG* expression was significantly reduced in the ACPs compared to the adenomas ($p < 0.001$; table 3; fig. 1). *OCT4* (also known as *POU5F1*) expression was elevated in all tumours, and its expression was higher in the somatotropinomas, corticotropinomas, and non-functioning adenomas than in the ACPs ($p < 0.001$, $p < 0.05$, $p < 0.05$, respectively; table 3; fig. 1). *DLL4* expression was high in all tumours, and its expression differed significantly only between the ACPs and corticotropinomas ($p < 0.05$; table 3; fig. 1). *ABCG2* expression did not significantly differ between the tumour types analysed, and it was down-regulated in all tumours (table 3; fig. 1). *NOTCH2* expression was within the normal range in the ACPs and was significantly decreased in the adenomas ($p < 0.001$; table 3; fig. 1). Increased *MKI67* expression was observed in all tumour types analysed (table 3; fig. 1). The highest *MKI67* expression was detected in the corticotropinomas and ACPs with fold changes of 4.03 and 3.91, respectively.

No different pattern expression of all analysed stem cell markers was observed in patients under medication compared with those without medication (online suppl. table 1, see www.karger.com/doi/10.1159/000446072).

Clinical Follow-Up of the Patients

Taking into account the tumour type, as well as tumour resection and recurrence, we found that the 3 patients with ACP recurrence had the highest SOX9 expression in the ACP group (table 4).

Discussion

Previous studies have suggested that the pituitary contains stem/progenitor cells that are responsible for cell renewal and maintenance of the gland in adults [2–4, 21, 22]. The same mechanism has also been suggested to give rise to tumours characterized by uncontrolled cell division. Notably, previous studies have demonstrated the presence of stem cells through SOX2 and SOX9 expression in pituitary adenoma and ACP [5, 6, 17, 23].

In the present study, we examined the differential expression of several stem cell markers in pituitary tumours (functional and non-functional) and ACP using RT-qPCR and immunohistochemistry. We found that these tumour types presented with different stem cell marker profiles, with a large number of undifferentiated stem cell markers expressed in ACP.

Table 4. Clinical follow-up of patients with ACP and relative SOX9 expression

Patient	Sex	Age, years	SOX9	Recurrence
25	M	21	2.74	No
26	M	9	3.44	No
27	F	17	2.64	Lost
28	F	3	2.69	Lost
29	F	11	0.04	No
30	F	9	3.23	Lost
31	F	7	4.17	Yes
32	F	10	4.23	Yes
33	F	14	4.29	Yes

Lost = Patient lost to follow-up.

It has previously been described that *CTNNB1* mutation in residues located in exon 3 disrupt the mechanism of its ubiquitination and degradation leading the β -catenin to enter into the nucleus and activate Wnt signaling related to proliferation. In our cohort all tumor samples had DNA extracted from paraffin and 5 out of 9 (55%) harbored mutation in the exon 3 of *CTNNB1* using Sanger sequencing. Immunohistochemical staining for β -catenin expression showed a nuclear staining pattern in all analyzed samples with and without mutation [13]. Preda et al. [24] observed *CTNNB1* mutation in 4 specific residues of exon 3 in 53% ACP samples also using paraffin-extracted DNA, and all samples have 100% cytosolic/nuclear β -catenin staining. In the literature, reported frequency of *CTNNB1* in exon 3 varies widely from 16 to 100% [25, 26] when using Sanger sequencing. The whole exome approach, however, has shown mutations in *CTNNB1* in 11 out of 12 (96%) ACP samples [27], suggesting that the Sanger method is not sufficiently sensitive to detect the presence of a mutation in all samples.

SOX9, a marker of transit-amplifying progenitors, has been demonstrated to be highly expressed in human ACP tumours and in the pituitaries of double heterozygous *Hesx1*^{Cre/+}; *Ctnnb1*^{+/-lox(ex3)} mice, a model created to study ACP [17]. In another human study, SOX9 showed a diffuse pattern of expression in the three epithelial layers in ACP, with the greatest expression in the nuclei of basal epithelial cells and diminishing intensity towards squamous cells [16]. In the present study, we found increased SOX9 mRNA expression in ACP compared to pituitary adenoma. SOX9 nuclear immunostaining was observed in the palisaded basal cell layer of the epithelium and, surprisingly, in the stellate stromal cells under

the epithelium. In addition, we found that 3 patients with ACP relapse had the highest *SOX9* expression levels. This finding suggests the involvement of pituitary progenitor/stem cells in tumour progression and recurrence. Overexpression of *SHH* has previously been described in ACP [9, 14]. Since *SOX9* is a downstream target of *SHH* in other systems such as the nervous system and skin [28, 29], it is possible that *SOX9* overexpression found in our samples could also be secondary to *SHH* overexpression.

CD44, a cell surface glycoprotein, has been implicated in cell adhesion, proliferation and metastatic tumour growth, and expression of this marker has been associated with the degree of cellular differentiation [30, 31]. We detected *CD44* overexpression in all of the ACPs but not in the adenomas. Hölsken et al. [18] found no differential or increased expression of *CD44* in ACP; however, in our study, we observed the greatest *CD44* expression in ACP. We believe that this discrepancy between studies is mainly due to differences in the control samples used; our study used normal pituitary tissues as a control, whereas the previous study used tissues from a pool of ACP cases. High *CD44* expression levels have previously been described in metastatic brain tumours and glioblastoma multiforme, whereas increased levels have not been detected in pituitary adenomas [32], not even in those with highly invasive behaviours [33, 34].

SOX2 is required for the maintenance of stem cell populations in different tissues, and it is expressed in a population of cells in the adult pituitary gland that have several characteristics of stem cells [22, 35]. In the present study, no significant change in *SOX2* expression was detected among the different tumours or in comparison with the normal pituitary samples. However, *SOX2* expression was highly variable among the ACP samples. The same *SOX2* variability was observed at the protein level in immunohistochemistry analysis. Gaston-Massuet et al. [17] did not observe differential *SOX2* expression in ACP; however, Garcia-Lavandeira et al. [16] demonstrated positive *SOX2* immunostaining in ACP with a variability in its expression among samples, similar to our findings. Andoniadou et al. [8] have shown that CP tumour cells do not originate from *SOX2*-positive cells but rather from adjacent cells, suggesting a paracrine mechanism of action.

An elevated expression of *DLL4*, a vascularization initiator stem cell marker, and *POU5F1/OCT4* were observed in all ACPs and pituitary adenomas compared with normal pituitary cells, indicating that both tumour

types originate from a pituitary cell at some point between totipotency and differentiation. *POU5F1/OCT4* expression has been reported to be increased at the protein level in ACP [16]. In the present study, we confirmed that *POU5F1/OCT4* is overexpressed at the RNA level in all ACPs and in functioning and non-functioning adenomas.

In addition, Notch signalling plays an essential role in early embryonic development of the pituitary gland. Among the Notch receptors, *NOTCH2* is one of two receptors expressed in the embryonic gland [36, 37]. Studies of transgenic mice have shown that *Notch2* expression is essential for maintaining embryonic progenitor cells in an undifferentiated state and that its down-regulation is a prerequisite for initializing the transition to differentiation. Remarkably, in the present study, this receptor was down-regulated in the pituitary adenomas compared with the more undifferentiated ACP tumours.

ABCG2, a member of the ATP-binding cassette G2 subfamily, is a gene that promotes oral cancer development and multidrug resistance when it is overexpressed in tumour cells [38–40]. In addition, *NANOG* is a member of the homeobox family of DNA binding transcription factors that plays an important role in the maintenance of pluripotency and in the self-renewal of human embryonic stem cells [41]. Our results showed that *ABCG2* and *NANOG* were not overexpressed, suggesting that they are not associated with the aggressive behaviour of the ACPs.

Pituitary tumours with invasive behaviour have been demonstrated to exhibit increased *MKI67* expression [42]. However, another study has shown considerable overlap of *MKI67* labelling in non-invasive and invasive adenomas as well as in adenomas and pituitary carcinomas [43]. We found that *MKI67* was more expressed in corticotropinomas than in the other pituitary adenomas, which is intriguing because corticotropinomas are the smallest in size.

Some of our patients were under treatment (cabergoline, ketoconazole, and/or octreotide) at the time of surgery, which could have altered the tumour cell viabilities. Cabergoline has been reported to decrease cell viability [44]. Ketoconazole has been demonstrated to decrease cell viability and also to promote the increased expression of apoptosis-related genes and cell cycle inhibitors in immortalized cell lines [45]. Stem cell expression patterns in patients taking these drugs have not been reported, and the low number of patients under medication in the present study is too small to obtain a definitive conclusion.

In conclusion, the results of our study have confirmed the presence of stem/progenitor cell markers in human pituitary tumours as well as the different expression patterns in ACP compared to adenoma. In particular, the ACPs expressed a larger number of stem cell markers compared to the adenomas. Significant overexpression of *SOX9* and *CD44* was observed in the ACP tumours. These results suggest that ACP originates from a more undifferentiated cluster of cells than pituitary adenoma. The findings of increased tumour recurrence among the CPs with high *SOX9* expression and *SOX9* immunolocalization to the underlying stromal tissue suggest that this factor may be associated with susceptibility to tumour recurrence.

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Disclosure Statement

The authors indicate that there are no potential conflicts of interest.

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