Why don't corticotroph tumors always produce Cushing's disease?

A García-Martínez[1], D A Cano[2], A Flores-Martínez[2], J Gil[3], M Puig-Domingo[3], S M Webb[4], A Soto-Moreno[2] and **A Picó[5]**

[1]Research Laboratory, Alicante General University Hospital-Institute for Health and Biomedical Research (ISABIAL), Alicante, Spain, [2]Unidad de Gestión de Endocrinología y Nutrición, Instituto de Biomedicina de Sevilla (IBiS), Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla, Sevilla, Spain, [3]Department of Endocrinology and Nutrition, University Hospital and Health Science Research Institute Germans Trias i Pujol, Badalona, Spain, [4]Endocrinology/Medicine Department, Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER, Unidad 747), ISCIII, Research Center for Pituitary Diseases, Hospital Sant Pau, IIB-Sant Pau, and Universitat Autònoma de Barcelona (UAB), Barcelona, Spain, and [5]Endocrinology Department, Alicante General University Hospital-ISABIAL, Miguel Hernández University, CIBERER, Alicante, Spain

Correspondence should be addressed to A Picó
Email
antonio.pico@umh.es

Abstract

Objective: Silent corticotroph tumors are a pituitary neuroendocrine tumor subtype of corticotroph lineage that do not clinically express Cushing's disease. The silencing of this type of tumor is not fully understood. The aim of the present study was to delve into the lack of secretory activity, studying the post-transcriptional and post-translational regulation of POMC/ACTH in a series of molecularly identified functioning and silent corticotroph tumors.
Design: We analyzed 24 silent corticotroph, 23 functioning corticotroph and 25 silent gonadotroph tumors.
Methods: We used Sanger sequencing, quantitative real-time PCR and Western blot to analyze genetic alterations in *POMC*, gene expression of *TBX19*, *NEUROD1*, *POMC*, *PCSK1*, *PCSK2*, *CPE* and *PAM* and protein expression of POMC, PC1/3, PC2, CPE and PAM.
Results: We found different polymorphisms in the *POMC* gene of corticotroph tumors, some of them related to deficiency of proopiomelanocortin. Silent corticotroph tumors showed lower PC1/3 gene and protein expression than functioning ones, especially compared to micro-functioning corticotroph tumors (all $P < 0.05$). Moreover, we found a positive correlation between PC2 and CPE gene and protein expression (rho ≥ 0.670, $P < 0.009$) in silent corticotroph tumors compared with functioning ones.
Conclusions: By studying the post-transcriptional and post-translational processing of *POMC* and ACTH, respectively, in a large series of silent and functioning corticotroph tumors, we found that the lack of secretory activity of these tumors is related to an impaired processing of *POMC* and a high degradation of ACTH, with the macro-functioning corticotroph tumor behaving as an intermediate state between micro-functioning and silent corticotroph tumors.

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Introduction

The application of immunohistochemistry to the identification of pituitary tumors led to the discovery of some tumors that are unaccompanied by a recognizable clinical endocrine syndrome. These tumors are known as silent pituitary adenomas (1, 2) or silent pituitary neuroendocrine tumors (silent PitNETs) as has been recently established (3). Silent corticotroph tumors (CTs) comprise up to 20% of silent PitNETs and approximately 40% of all CTs (4). They have been reported as more aggressive and recurrent than functioning CTs (4, 5), although there are conflicting results about their behavior compared with other silent tumors. Indeed, a recent

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meta-analysis carried out by Fountas *et al*. (6) concludes that there is no evidence that silent CTs are associated with a higher risk of recurrence compared to other silent subtypes. Both functioning and silent CTs arise from cells of Tpit lineage (Tbox family member *TBX19*, transcription factor involved in the differentiation of corticotroph cells) and usually produce and secrete adrenocorticotropic hormone (ACTH) (7).

While the clinical and biochemical characteristics of silent CTs are discernible, the mechanisms of silencing of these tumors are not fully understood. The most studied hypothesis relates to the post-transcriptional regulation of *POMC* and post-translational regulation of ACTH. Some authors (8, 9, 10, 11, 12) have suggested that the transcription factors and convertases involved in the processing of *POMC* and degradation of ACTH are implicated in the lack of secretory activity that gives rise to the absence of Cushing's disease. *TBX19* (Tpit) and *NEUROD1* are transcription factors expressed in CT cells that contribute to the corticotroph-specific *POMC* transcription (13, 14). The proconvertase PC1/3 is the next factor involved in the ACTH secretion pathway. PC1/3, a convertase encoded by *PCSK1* gene, is found in corticotroph cells of the anterior pituitary and in the melanotroph cells of the intermediate pituitary. It is responsible for the processing of *POMC* into biologically active ACTH and other peptides like joining peptide, beta-lipoprotein, amino terminal and gamma-lipoprotein (13, 15, 16). The proconvertase PC2, encoded by the *PCSK2* gene, is an endoproteolytic enzyme responsible for the processing of biologically active ACTH. Unlike PC1/3, PC2 is only present in the intermediate lobe pituitary cells, where it processes mature ACTH (1–39) to produce corticotropin-like intermediate lobe peptide (CLIP) and a smaller ACTH (1–17) peptide (16, 17). Finally, the ACTH fragments are cleaved to deacetyl alpha-MSH (da-MSH) by carboxypeptidase E (CPE) and PAM (13).

All the previous research performed on the silencing mechanisms of CTs has pivoted on the identification by immunochemistry (IHC) of these tumors. But some PitNETs of Tpit lineage do not secrete ACTH identifiable by IHC (18). Indeed, we have recently demonstrated in two large and independent series of PitNETS a poor concordance between the molecular and IHC identification of silent CTs (19, 20). Therefore, the aim of the present study is to analyze the complete pathway of *POMC* processing and ACTH degradation in a large series of molecularly identified CTs. Moreover, we quantified the gene and protein expression of POMC, PC1/3, PC2, CPE and PAM, sequenced the *POMC* gene and quantified the gene expression of transcription factors involved in the corticotroph differentiation.

Methods

Patients and samples

From our collection of PitNETs, we selected 24 silent CTs, 23 functioning CTs and 25 silent gonadotroph tumors (GTs; control group) with abundant, good-quality genetic material. Clinical, immunohistochemical and molecular data were collected anonymously from the Spanish Molecular Registry of PitNETs (REMAH) database, which is part of a Spanish multicenter project (21). Different PitNET subtypes were identified based on the relative gene expression of the pituitary-specific hormone genes and following the criteria previously published by our group (19, 20). Namely, we identified CTs when the gene expression of *POMC*, arginine vasopressin receptor 1B (*AVPR1B*) and corticotropin-releasing hormone receptor 1 (*CRHR1*) were dominant with respect to other pituitary-specific hormone genes. We defined micro/macroadenomas and invasiveness according to Hardy's classification and cavernous sinus invasion (CSI), respectively. The demographic, clinical, biochemical, immunohistochemical and molecular characteristics of the patients are shown in Table 1 and Supplementary Table 1 (see section on supplementary data given at the end of this article). There was some discordance between the molecular and IHC analysis of the different subtypes, especially in silent CTs and silent GTs. This can be attributed to the fact that the IHC studies were performed in different pathology departments belonging to the six participating hospitals, whereas the molecular study was centralized in the research laboratory of the Alicante General University Hospital. The low concordance between the molecular and IHC identification of tumors has been previously described by our group (19, 20).

The study complies with the Declaration of Helsinki and other applicable laws. The study was approved by the Clinical Research Ethics Committee of the General University Hospital of Alicante. None of the donors came from a vulnerable population, and all donors or relatives freely provided written informed consent.

DNA and RNA extraction and cDNA synthesis

All molecular studies were centralized in the Alicante General University Hospital Research Institute (ISABIAL)

Table 1 Characteristics of the studied cohort.

Variables	Frequency	
	n	%
Sex		
Men	26	36.1
Women	46	63.9
Tumor size		
Macro	60	83.3
Micro	12	16.7
Invasiveness		
CSI	34	47.2
No CSI	38	52.8
Molecular		
Silent CTs	24	33.4
Functioning CTs	23	31.9
Silent GTs	25	34.7
Age (years; mean ± S.D.)	50.3 ± 15.9	

CTs, corticotroph tumors; CSI, cavernous sinus invasiveness; GTs, gonadotroph tumors; S.D., standard deviation.

laboratory, with the exception of the *POMC* sequencing, which took place in IMEGEN (Valencia). Quantification of protein expression by Western blot was performed in the Institute of Biomedicine of Sevilla.

All samples were preserved immediately after surgery in RNAlater solution at 4°C for 24 h and then stored at −20°C. The biological samples were disintegrated in the TissueLyser (Qiagen). We used the AllPrep DNA-RNA-Protein kit (Qiagen) for manual DNA and RNA extraction and measured their concentration and purity in the NanoDrop spectrophotometer (Thermo Scientific).

For each retrotranscription reaction, we used 2 µg of RNA in a total volume of 20 µL, employing the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems).

Sanger sequencing

Sanger sequencing was performed in tumor samples of 18 silent CTs and 21 functioning CTs, due to the quantity and quality of the available genetic material. The methodology of the Sequencing Service of the Institute of Genomic Medicine (IMEGEN, Valencia, Spain) was followed. Specific primers were designed to amplify the four exons of the *POMC* gene. To obtain the sequence of each exon, a pair of primers was used, except for exon 4, for which two pairs of overlapping primers were designed. The sequence of the primers were *POMC*-1-forward: 5′-CAACGCCATCCATAATTAAGTTCTTC-3′; *POMC*-1-reverse: 5′-TCCCAAGACCTCCTAGCAAGC-3′; *POMC*-2-forward: AAATCCAAAATGATTGTAGGGTT GG-3′; *POMC*-2-reverse: 5′-TCAGTTCTTTGAAA TCTGGCTCATC-3′; *POMC*-3-forward: 5′-GTGTTGTTAATGTTGGCTCAAGGTC-3′; *POMC*-3-reverse: 5′-CCCTGATTTCTTCTCCCATTTCTTA-3′; *POMC*-4a-forward: 5′-CTTTCTTGGAGCCACTCCTTTATGT-3′; *POMC*-4a-reverse: 5′-GTCAGCTCCCTCTTGAACTCCA-3′; *POMC*-4b-forward: 5′-GAGCAGCCTCTGACCGAGAA-3′; *POMC*-4b-reverse: 5′-AGGTGGATGTGAAATTTGAAAG GTT-3′. We used the KAPA2G Fast HotStart ReadyMix with dye kit (Kapa Biosystems). In the case of exon 4b, betaine solution 5M and PCR Reagent (SIGMA; Ref:B0300-1VL) were also added. The PCR reaction was carried out at 95°C for 2 min, 40 cycles of 95°C for 15 s, 55°C for 15 s and 72°C for 10 s and finally, 72°C for 1 min. Purification of PCR products was carried out with Acroprep Advance (Pall corporation; Ref:8035). A total of 1 µL of the purified PCR product was used as DNA mold for the sequence reaction using the BigDye Terminator Sequencing kit V3.1 (Applied Biosystems). Sequencing was carried out on an ABI3730 XL sequencer from Applied Biosystems.

Quantitative real-time polymerase chain reaction (qRT-PCR)

We performed qRT-PCR following the manufacturer's instructions in the 7500 Fast Real-Time PCR system (Life Technologies) and used TaqMan Fast Advanced PCR Master Mix and assays based on hydrolysis probes (TaqMan Gene Expression Assays, Life Technologies). We selected the following assays: *POMC* (Hs01596743_m1), *AVPR1B* (Hs00949767_m1), *CRHR1* (Hs00366363_m1), *TBX19* (Hs00193027_m1), *NEUROD1* (Hs01922995_s1), *PCSK1* (Hs01026107_m1), *PCSK2* (Hs00159922_m1) and *CPE* (Hs00175676_m1), and *PAM* (Hs00168596_m1). Reference genes used were *PGK1* (Hs00943178_g1), *TBP* (Hs00427620_m1) and *MRPL19* (Hs00608519_m1). A pool of RNA from nine normal pituitary samples obtained from autopsies served as a calibrator. All samples were analyzed in duplicate. The relative differences in gene expression were expressed as fold change and were obtained with the $2^{-\Delta\Delta Ct}$ method (SDS software, Applied Biosystems).

Western blot

Protein was extracted from tumor samples using the AllPrep DNA-RNA-Protein kit (Qiagen). Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). We loaded 20 µg of total protein on 8% SDS-PAGE for separation and subsequently transferred it to PVDF membranes. The membranes were then processed for immunoblotting

against the following antibodies: anti-PC1/3 (1:1000, 11914) and anti-PC2 (1:1000, 14013) from Cell Signaling Technology and anti-POMC (1:2000, PA5-18368), anti-PAM (1:250, PA5-59929) and anti-Carboxypeptidase E (1:2000, PA5-28376) from Thermo Fisher Scientific. Anti-β-actin (1:1000, Abcam ab8226) served as protein loading control. HRP-conjugated secondary antibodies from Jackson ImmunoResearch were used. Immunoblots were revealed with Clarity Western ECL Substrate (Bio-Rad) and visualized using an ImageQuant LAS4000 Mini Gold imager (GE Healthcare). Band intensity was analyzed using ImageJ software. Relative protein expression (intensity relative to the amount of loaded protein, determined by the intensity of β-actin) was also determined. Protein quantification of Tpit and NEUROD1 was not performed due to the limited amount of material available.

Statistical analysis

Qualitative variables (including PitNET subtypes) were expressed as absolute and relative frequencies. Participants' age and tumor diameter were expressed as mean ± standard deviation (s.d.). We used the Shapiro–Wilk test to investigate normality in the distribution of the molecular variables (fold change and relative protein expression). To compare qualitative and quantitative variables, we used the Kruskal–Wallis and Mann–Whitney *U* test or the Student's *t* and ANOVA tests, as appropriate. Spearman and Pearson correlation tests were used to compare quantitative variables. *P* values of less than 0.05 were considered to be statistically significant. Statistical analysis was performed with SPSS 24.0 software (IBM Software; Miguel Hernandez University, Alicante, Spain).

Results

Sequencing of *POMC*

Sanger sequencing of *POMC* in tumor samples of 18 silent CTs and 21 functioning CTs showed some single nucleotide and deletion-insertion variants. We found some of these polymorphisms only in functioning CTs, some of them only in silent CTs and some of them in both functioning and silent CTs. Some samples showed more than one polymorphism. Table 2 summarizes the frequency of polymorphisms for both the whole series and by subtypes. Supplementary Table 2 shows these results in detail.

Among the polymorphisms observed, five were associated with proopiomelanocortin deficiency and monogenic non-syndromic obesity (rs1042571, rs10654394, rs8192605, rs28930368 and rs2071345). The variation class, minor allele frequency, clinical significance and conditions are summarized in Supplementary Table 3. Of the samples that presented the rs1009388 and the rs13404336 polymorphism, 81.8 and 100%, respectively, were functioning CTs ($P=0.030$ and $P=0.011$, respectively). No associations were found between the presence of the different polymorphisms and clinical-radiological variables (age, tumor size and invasiveness; all $P>0.05$) in the overall series or by CT subtypes.

Gene expression of *POMC* and hypothalamic hormone receptors

POMC, *AVPR1B* and *CRHR1* gene expression was significantly higher in CTs, both functioning and silent, than in silent GTs (all $P<0.001$). Silent CTs showed higher expression of only *AVPR1B* compared to functioning CTs ($P=0.044$) (Table 3).

Table 2 Frequency of polymorphisms in the global series and by subtypes. Data are presented as *n* (%).

RefSNP	Overall (*n* = 39)	Silent CTs (*n* = 18)	Functioning CTs (*n* = 21)
rs6713532	12 (30.8)	4 (22.2)	8 (38.1)
rs1009388	11 (28.2)	2 (11.1)	9 (42.9)
rs13404336	5 (12.8)	0 (0.0)	5 (23.8)
rs1042571	13 (33.3)	7 (38.9)	6 (28.6)
rs8192605	1 (2.6)	0 (0.0)	1 (4.8)
rs10654394	3 (7.7)	2 (11.1)	1 (4.8)
rs6713396	1 (2.6)	1 (5.6)	0 (0.0)
rs28930368	1 (2.6)	1 (5.6)	0 (0.0)
rs2071345	1 (2.6)	1 (5.6)	0 (0.0)
G.12086_12104dup	1 (2.6)	1 (5.6)	0 (0.0)

CTs, corticotroph tumors; RefSNP, reference single nucleotide polymorphism.

Table 3 Fold change (FC) means and s.d. of *POMC* and hypothalamic hormone receptors in functioning CTs, silent CTs and silent GTs.

Gene	FC, mean ± s.d.		
	Functioning CTs	Silent CTs	Silent GTs
POMC	5.94 ± 7.17*	4.59 ± 5.37*	0.00 ± 0.01
AVPR1B	24.83 ± 23.13*,†	51.27 ± 45.96*	0.06 ± 0.08
CRHR1	23.45 ± 23.18*	23.32 ± 30.01*	0.19 ± 0.21

*$P < 0.001$ compared with silent GTs; †$P = 0.044$ compared with silent CTs. CTs, corticotroph tumors; FC, fold change; GTs, gonadotroph tumors; SD, standard deviation.

Micro-functioning CTs showed higher expression of *CRHR1* (34.55 ± 25.51) than macro-functioning CTs (13.27 ± 15.72) ($P=0.019$), without significant differences compared to silent CTs (23.32 ± 30.01). By contrast, the gene expression of *AVPR1B* was higher in silent CTs than in macro-functioning CTs (51.27 ± 45.96 vs 20.01 ± 24.54; $P=0.037$) but similar to micro-functioning CTs (29.99 ± 21.40). There were no differences in *POMC* expression between the different CT subtypes.

Gene expression of transcription factors and convertases involved in *POMC* processing

TBX19 expression was significantly higher in functioning and silent CTs than in silent GTs (all $P<0.001$), although *NEUROD1* expression was not (Fig. 1).

Both functioning and silent CTs showed higher expression of *PCSK1* ($P<0.001$ and $P=0.031$, respectively) than silent GTs. Conversely, the expression of *PCSK2* and *PAM* was lower in functioning ($P<0.001$ and $P=0.001$, respectively) and silent CTs ($P<0.001$ and $P=0.035$, respectively) (Fig. 1).

Only micro-functioning CTs showed higher expression of *PCSK1* than silent CTs ($P=0.001$), with no differences in the rest of convertases nor in the transcription factors studied (Table 4).

Finally, there were no differences in *CPE* expression between silent GTs, functioning CTs and silent CTs or between micro- and macro-functioning CTs and silent CTs (Fig. 1 and Table 4).

Protein expression of POMC and convertases involved in the processing of *POMC*

Representative immunoblotting analysis of POMC expression and the convertases of corticotroph lineage are shown in Fig. 2. Similar to gene expression studies, we found differences in protein production among the different PitNET subtypes studied (Fig. 3).

Higher POMC expression was observed in functioning CTs than in silent GTs ($P<0.001$) and silent CTs (only a trend, $P=0.088$) (Fig. 3). No differences were observed by size (data not shown).

Similar to the gene expression pattern, both functioning and silent CTs showed higher expression of PC1/3 than silent GTs ($P=0.004$ and $P=0.020$, respectively; Fig. 3), and micro-functioning CTs showed higher PC1/3 protein expression than silent ones ($P=0.028$; data not shown). Moreover, PC2 showed higher expression in silent GTs than in functioning and silent CTs ($P<0.001$ and $P=0.002$, respectively; Fig. 3).

However, CPE was more expressed in CT tumors, both functioning and silent, than in silent GTs ($P=0.006$ and $P=0.002$, respectively), despite the absence of differences

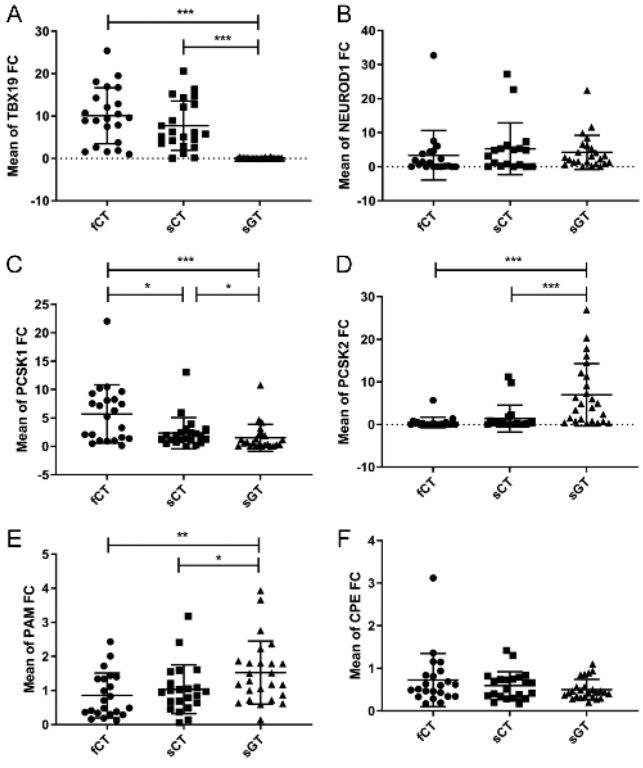

Figure 1
Gene expression of transcription factors and convertases of corticotroph lineage between subtypes. (A) Gene expression of *TBX19*; (B) gene expression of *NEUROD1*; (C) gene expression of *PCSK1*; (D) gene expression of *PCSK2*; (E) gene expression of *PAM*; (F) gene expression of *CPE*. FC, fold change; fCT, functioning corticotroph tumors; sCT, silent corticotroph tumors; sGT, silent gonadotroph tumors. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

Table 4 Fold change (FC) means and S.D. of transcription factors and convertases of corticotroph lineage in macro-functioning CTs, micro-functioning CTs and silent CTs.

Gene	FC, mean ± S.D.		
	Macro-functioning CTs	Micro-functioning CTs	Silent CTs
TBX19	8.499 ± 5.116	11.661 ± 7.746	7.73 ± 5.803
NEUROD1	19.384 ± 46.191	1.927 ± 2.402	38.436 ± 144.648
PCSK1	5.432 ± 6.880	6.011 ± 2.777*	2.341 ± 2.746
PCSK2	0.876 ± 1.749	0.115 ± 0.154	1.419 ± 3.173
PAM	0.682 ± 0.737	1.029 ± 0.545	1.037 ± 0.715
CPE	0.774 ± 0.840	0.673 ± 0.327	0.593 ± 0.328

*$P = 0.001$ compared with silent CT.
CTs, corticotroph tumors; FC, fold change; SD, standard deviation.

in the corresponding gene expression studies. Finally, and also different from gene expression results, we did not find differences in the expression of PAM between subtypes (Fig. 3).

Correlation between gene and protein expression and demographic and radiological variables

Table 5 shows the significant correlations between gene and protein expression in the tumors. The correlation *PCSK1*-PC1/3 and POMC-POMC were stronger in functioning CTs than in silent CTs. Conversely, the correlations *PCSK2*-PC2 and CPE-CPE were stronger in silent compared to functioning CTs. No correlation was found between gene and protein expression with any of the demographic and radiological variables, either in the overall series or by subtypes (all $P > 0.05$).

Discussion

In this study, we found decreased *POMC* expression and processing and higher ACTH degradation in silent compared to functioning CTs. Moreover, we sequenced the *POMC* gene in DNA obtained from tumor samples and used qRT-PCR and Western blot techniques in a large series of functioning and silent CTs.

We identified the different subtypes of PitNETs on the basis of their molecular, rather than IHC, characteristics. Our group recently described the molecular identification of PitNET subtypes based on the relative expression of the pituitary-specific hormone genes. However, the concordance between molecular and IHC identification was strong in functioning tumors and weak in non-functioning tumors, likely because of the high percentage of null-cell tumors identified by the IHC studies.

As this is the first time that the CT subtypes have been identified according to their pituitary-specific hormone

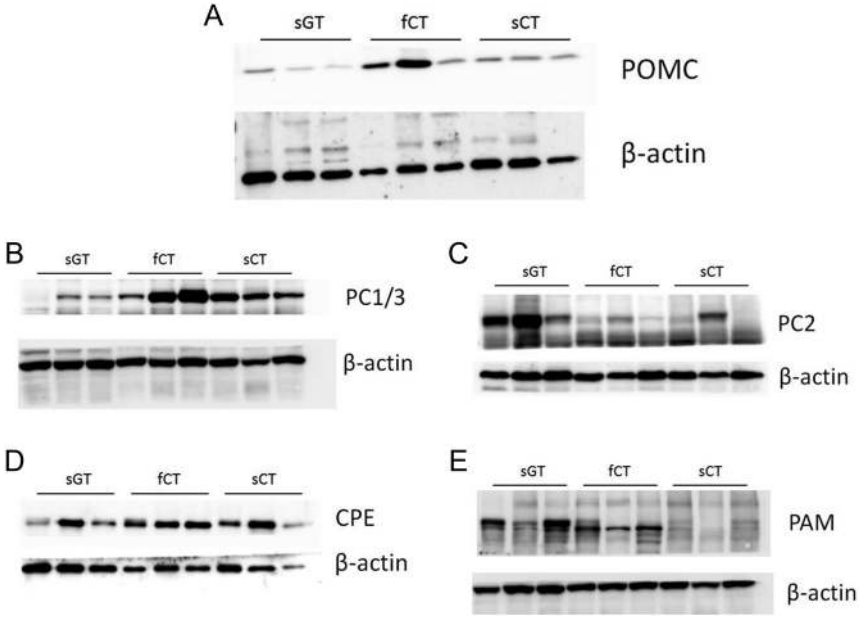

Figure 2
Western blot analysis of POMC (A), PC1/3 (B), PC2 (C), CPE (D) and PAM (E) expression. fCT, functioning corticotroph tumors; sCT, silent corticotroph tumors; sGT, silent gonadotroph tumors.

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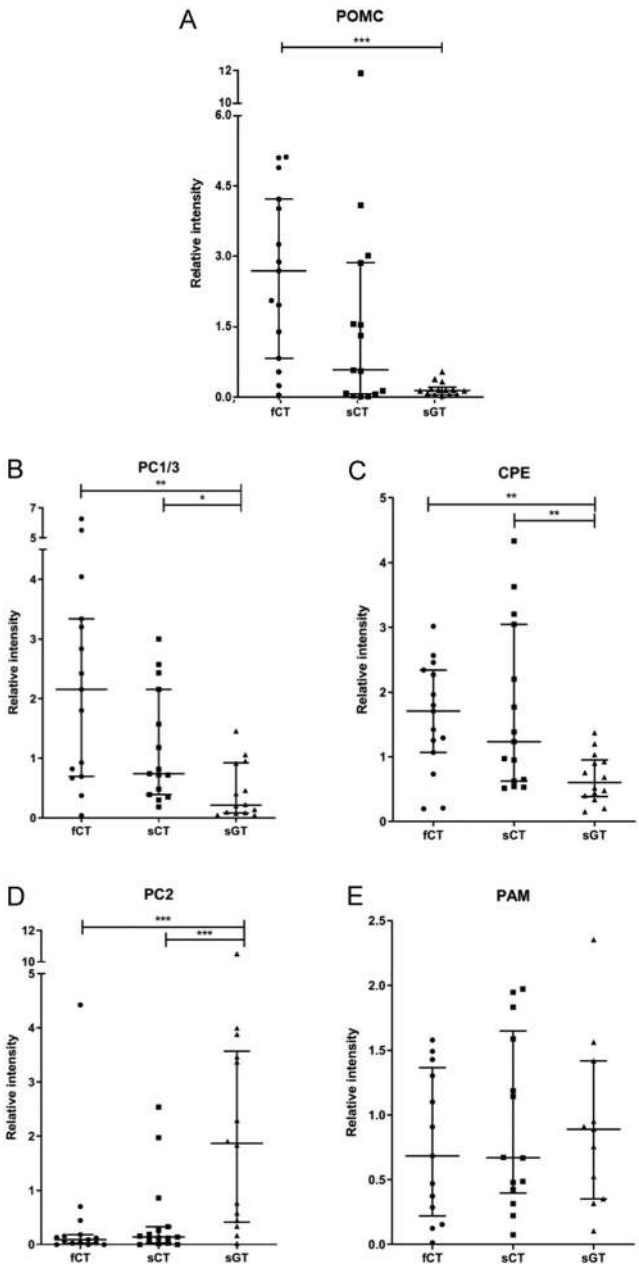

Figure 3

Quantification of protein expression between tumor subtypes, as analyzed by Western blot. Relative protein expression was normalized to β-actin levels. (A) Protein expression of POMC; (B) protein expression of PC1/3; (C) protein expression of CPE; (D) protein expression of PC2; (E) protein expression of PAM. fCT, functioning corticotroph tumors; sCT, silent corticotroph tumors; sGT, silent gonadotroph tumors. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

gene expression (*POMC*, *CHRH* and *AVPR1*) instead of their IHC pattern (ACTH), this could imply a bias in the comparison of our results with those of other studies. As shown in Supplementary Table 1 as well as in previous studies reported by our group (19, 20), IHC data were very heterogeneous among the different participating pathology departments, likely due to differences in the antibodies used, the pathologists responsible for interpreting the results, and the different ways of expressing them (% vs +).

The first hypothesis on the silencing mechanisms of CTs arose decades ago. In 1978, Kovacs *et al*. (22) observed that silent CT cells showed dark lysosomes that could degrade ACTH before it could be secreted. Later, in 1990, Nagaya *et al*. carried out a ribonuclease mapping analysis, studying the possibility that a defective *POMC* was responsible for the silencing of CTs. However, the authors did not find any abnormality in the size or coding sequence of *POMC* (23).

In the present study, we found two polymorphisms associated with functioning CTs and five polymorphisms associated with proopiomelanocortin deficiency, four of which were only present in silent CTs. However, the clinical significance of these polymorphisms is unclear, and more studies are needed.

More recently, Tateno *et al*. analyzed several genes involved in the transcription, processing and secretion of *POMC* in 12 functioning CTs, 8 silent CTs and 15 other non-functioning PitNETs, finding that *POMC* expression was higher in CTs than in non-CT non-functioning PitNETs. Similarly, Nishioka *et al*. found higher *POMC* mRNA levels in ACTH-negative and Tpit-positive tumors than in silent GTs (18). In agreement with these results, *POMC* expression was lower in silent GTs than in CTs (both functioning and silent) in our series. Moreover, and despite the absence of differences in *POMC* gene expression, silent CTs also showed lower POMC protein expression than functioning ones. Indeed, the correlation gene–protein of POMC was lost in silent CTs but not in functioning ones.

As expected, CTs expressed more *TBX19* than silent GTs did. We observed a tendency of micro-functioning CTs to express more *TBX19* than both macro-functioning and silent ones, although the differences were not statistically significant. Other authors have reported similar results. Tateno *et al*. observed higher *TBX19* levels in CTs than in non-CT non-functioning PitNETs, although there are no differences between CT subtypes (12). Similarly, Tabuchi *et al*. (24) observed higher *TBX19* gene expression in seven functioning CTs than in six silent ones or ten other non-functioning PitNETs. These data suggest an impaired POMC post-transcriptional regulation in silent CTs, with the macro-functioning CTs behaving like an

Table 5 Spearman's rho correlation between gene and protein expression in the overall series and by subtypes.

Gene-Protein	Overall		Silent CTs		Functioning CTs		Silent GTs	
	rho	*P*	*rho*	*P*	*rho*	*P*	*rho*	*P*
POMC-POMC	0.505	0.01	−0.112	0.703	0.656	0.006	0.006	0.987
PCSK1-PC1/3	0.661	<0.001	0.400	0.156	0.547	0.043	0.600	0.067
PCSK2-PC2	0.840	<0.001	0.843	<0.001	0.555	0.040	0.818	0.002
CPE-CPE	0.571	<0.001	0.670	0.009	0.346	0.206	0.209	0.537
PAM-PAM	0.388	0.013	0.073	0.805	0.468	0.079	0.564	0.071

CTs, corticotroph tumors; GTs, gonadotroph tumors.

intermediate state between micro-functioning and silent CTs. The greater similarity in *NEUROD1* expression levels between macro-functioning and silent CTs compared to macro-functioning and micro-functioning CTs found in our study reinforces this hypothesis. Again, other authors, such as Tateno *et al*. (12), have reported a higher expression of *NEUROD1* in silent compared to micro-functioning CTs. In addition, Oyama *et al*. (14) and Ferreti *et al*. (25) showed an overexpression of *NEUROD1* in silent PitNETs. Although the pathogenesis of silent GTs is not the topic of the present study, Cooper *et al*. (26) have already suggested the existence of a mixed GT–CT subtype of PitNET.

On the other hand, *AVPR1b* and *CRHR1*, specific receptors for arginine vasopressin (AVP) and corticotropin-releasing hormones (CRH), respectively, have been reported to be overexpressed in functioning CTs compared to silent CTs and other non-functioning tumors (12). The higher *AVPR1b* mRNA expression in silent compared to functioning CTs observed in our series could be related to their larger size, since AVP is capable of activating pathways related to cell proliferation (12). In any case, our results suggest a low participation of AVP and CRH receptors in the lack of secretory activity of CTs or a bad processing of *AVPR1b* and *CRHR1* genes. Regrettably, we have not quantified the protein expression of these receptors. Moreover, differences among studies could also be attributed to the different series studied and to the different methodology used (TaqMan vs SYBR green).

Concerning *POMC* post-transcriptional regulation, the lower *PCSK1* and PC1/3 expression in silent vs functioning CTs found in our study suggests that *POMC* processing is also reduced in silent CTs. This could contribute to the absence of the clinical endocrine syndrome. Indeed, the correlation between gene and protein PC1/3 expression was lost in silent but not in functioning CTs. It could be possible that miRNAs are involved in this lack of correlation between gene (*PCSK1*) and protein (PC1/3) expression in silent CTs. For instance, a miRNA might target this gene and act on the mRNA, preventing their translation into

Table 6 Main published studies on the expression of PC1/3 in PitNETs.

Reference	PitNETs analyzed	*PCSK1* mRNA	PC1/3 protein	Techniques used
(12)	8 silent CTs 12 functioning CTs 15 silent PitNETs	Lower expression in silent CTs and silent PitNETs compared with functioning CTs	Not done	qRT-PCR (SYBR green)
(11)	6 silent CTs 24 silent PitNETs	Similar expression in silent CTs and silent PitNETs	Absence of expression in silent CTs	qRT-PCR (SYBR green)/IHC
(8)	14 silent CTs 15 macro-functioning CTs 21 micro-functioning CTs	Lower expression in silent CTs and macro-functioning CTs than in micro-functioning CTs		qRT-PCR (SYBR green) IHC
(15)	2 silent CTs 16 functioning CTs	Not done	Absence of expression in silent CTs	IHC
(32)	15 silent CTs 8 functioning CTs 8 silent PitNETs	Lower expression in silent CTs than in functioning CTs	Not done	qRT-PCR (SYBR green)
(33)	3 silent CTs 12 functioning CTs 28 silent PitNETs	Not done	Silent CTs and functioning CTs were all positive	IHC
Our study	24 silent CTs 23 functioning CTs 25 silent GTs	Lower expression in silent CT than in micro-functioning CTs		qRT-PCR (TaqMan probes) Western blot

CTs, corticotroph tumors; GTs, gonadotroph tumors; IHC, immunohistochemistry; qRT-PCR, quantitative real-time polymerase chain reaction.

protein. Moreover, although we selected the TaqMan probes with the best coverage (as recommended by the manufacturer), we do not study all the exons of the genes. On the other hand, the high intensity of a band of PC1/3 observed in functioning CTs could be related to a higher processing of PC1/3 in these tumors, as shown in pituitary cell lines (27, 28). In contrast to transcriptional regulation, translational regulation of *POMC* was more similar between macro- and micro-functioning CTs than between functioning and silent CTs. The expression of both *PCSK1* and PC1/3 in silent CTs has been extensively analyzed in the literature. The main published studies are summarized in Table 6. Our series constitutes the largest number of silent CTs reported to date. Furthermore, we have been the first to use qRT-PCR with TaqMan probes instead of SYBR green to quantify *PCSK1* expression. Also, Western blot was used instead of IHC to quantify PC1/3 expression. Despite the different technologies utilized, our results are similar to those previously reported, namely lower *PCSK1* and PC1/3 expression in silent CTs than in functioning CTs, thus reinforcing the hypothesis of impaired *POMC* processing in silent CTs. Indeed, Righi *et al*., studying a series of functioning and silent CTs, reported that the three cases of silent CTs that evolved during follow-up to functioning ones showed an increase in *PCSK1* and PC1/3 expression (9).

Regarding ACTH post-translation regulation, most authors have reported a decrease in ACTH degradation in CT tumors compared with other PitNET subtypes, without differences between functioning and silent CTs (12, 29). Our data agree with those previously reported, showing lower *PCSK2* and PC2 expressions in functioning and silent CTs than in silent GTs. However, we found a stronger correlation between *PCSK2* and PC2 expression in silent CTs than in functioning ones, and moreover, micro-functioning CTs exhibited lower *PCSK2* expression than macro-functioning CTs. These results suggest a more efficient degradation of ACTH in silent than in functioning CTs that might explain the absence of Cushing's disease in these tumors. Again, macro-functioning CTs displayed an intermediate pattern between micro-functioning CTs and silent CTs. Indeed, Raverot *et al*. (8) suggested that macro-functioning and silent CTs share common pathological pathways, different from those involved in the pathogenesis of micro-functioning ones.

Similar to *PCSK2* and PC2 expression, both PAM gene and protein expression was lower in CTs than in silent GTs in our study. Indeed, Steel *et al*. (30), studying the expression of PAM in 40 human pituitary tumors by *in situ* hybridization, also found that the gonadotroph and folliculo-stellate cells showed higher PAM staining intensity than corticotroph cells. Regrettably, the authors did not analyze the differences among the different subtypes of PitNETs included in their study. Although the differences were not statistically significant, there was a tendency to express more *PAM* mRNA in silent CTs than in functioning ones in our series. Since this enzyme is involved in the amidation reaction of ACTH for the synthesis of alpha-MSH, this suggests that silent CTs present a high capacity to amidate ACTH, thus contributing to the inactivation of the biological active peptide.

In contrast to previous results, the expression of CPE, another protein involved in the post-translational regulation of ACTH, was higher in CTs than in silent GTs, although the correlation between the gene and protein CPE expression was stronger in silent CTs than in functioning ones. Another study also found (31) higher immunopositivity of CPE in CTs than in silent GTs. While the meaning of these results should be clarified, it seems that CPE expression is related to the presence of ACTH and that its influence in the post-translational regulation is higher in silent CTs than in functioning ones, also contributing to the lack of secretory activity of these tumors.

In conclusion, by studying the post-transcriptional and post-translational processing of *POMC* and ACTH, respectively, in a large series of silent and functioning CTs, we have found that the lack of secretory activity of CTs is related to impaired *POMC* processing and increased ACTH degradation, with the macro-functioning CTs behaving as an intermediate state between micro-functioning and silent CTs.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/EJE-19-0338.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this study.

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