Landscape of somatic mutations in sporadic GH-secreting pituitary adenomas

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Abstract

Context: Alterations in the cAMP signaling pathway are common in hormonally active endocrine tumors. Somatic mutations at *GNAS* are causative in 30–40% of GH-secreting adenomas. Recently, mutations affecting the *USP8* and *PRKACA* gene have been reported in ACTH-secreting pituitary adenomas and cortisol-secreting adrenocortical adenomas respectively. However, the pathogenesis of many GH-secreting adenomas remains unclear.

Aim: Comprehensive genetic characterization of sporadic GH-secreting adenomas and identification of new driver mutations.

Design: Screening for somatic mutations was performed in 67 GH-secreting adenomas by targeted sequencing for *GNAS*, *PRKACA*, and *USP8* mutations ($n=31$) and next-generation exome sequencing ($n=36$).

Results: By targeted sequencing, known activating mutations in *GNAS* were detected in five cases (16.1%), while no somatic mutations were observed in both *PRKACA* and *USP8*. Whole-exome sequencing identified 132 protein-altering somatic mutations in 31/36 tumors with a median of three mutations per sample (range: 1–13). The only recurrent mutations have been observed in *GNAS* (31.4% of cases). However, seven genes involved in cAMP signaling pathway were affected in 14 of 36 samples and eight samples harbored variants in genes involved in the calcium signaling or metabolism. At the enrichment analysis, several altered genes resulted to be associated with developmental processes. No significant correlation between genetic alterations and the clinical data was observed.

Conclusion: This study provides a comprehensive analysis of somatic mutations in a large series of GH-secreting adenomas. No novel recurrent genetic alterations have been observed, but the data suggest that beside cAMP pathway, calcium signaling might be involved in the pathogenesis of these tumors.

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Introduction

Pituitary tumors represent ~15% of all primary intracranial lesions. Growth hormone (GH)-secreting pituitary adenomas are the second most frequent type of

hormone-producing pituitary tumors, after prolactin-secreting adenomas (1). Excessive secretion of GH causes gigantism during childhood and acromegaly in adults,

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with significant morbidity due to clinical complications involving cardiovascular, respiratory, and metabolic systems (2, 3).

The monoclonal origin of most pituitary adenomas indicates that these tumors derive from the replication of a single cell that acquired growth advantage. The latter has been suggested to result from genetic or epigenetic alterations, leading to activation of proto-oncogenes or inactivation of tumor suppressor genes (4, 5). However, despite intensive investigations, little is known about the genetic causes of pituitary adenomas. The only mutations identified to date in a significant proportion (30–40%) of sporadic GH-secreting adenomas occur in the gene encoding the α subunit of the stimulatory G-protein (*GNAS*) (6, 7, 8, 9). These somatic activating mutations (*gsp* mutations), found in codon 201 and 227, prevent hydrolysis of GTP, leading to a constitutive activation of the cAMP pathway, which in somatotrophs and in other endocrine cells acts as a mitogenic signal (10, 11). In somatotrophs, the *GNAS* transcript is expressed mainly from the maternal allele, due to tissue-specific paternal imprinting (12, 13). Consistently, *gsp* mutations in sporadic GH-secreting adenomas are found on the maternal allele (14), and partial loss of this imprinting is present in tumors negative for *gsp* mutations (15), further supporting the involvement of *GNAS* locus in pituitary tumorigenesis. So far, the screening for mutations in other G-protein subunits in pituitary tumors has given negative results (16, 17, 18).

Genetic alterations in other genes involved in cAMP signaling have been identified as the cause of other endocrine tumors. A reduced expression and/or function of the protein kinase A (PKA) regulatory subunit type I α (*PRKARIA*) due to loss-of-function mutations, leading to an abnormal cAMP pathway activation, causes GH-secreting pituitary adenomas in Carney complex, an autosomal dominant familial syndrome (19, 20). To date, mutations of *PRKARIA* gene have been rarely found in sporadic pituitary tumors (21, 22), although a reduced *PRKARIA* expression resulting from increased proteasomal degradation has been described in sporadic GH-secreting tumors (10). Reduced cAMP degradation caused by mutations in *PDE11A* and *PDE8B*, coding for members of the phosphodiesterase (PDE) family, have been involved in adrenocortical hyperplasia, adenomas, and cancer as well as in testicular germ cell tumors (23, 24). However, genetic variants of *PDE11A4* contribute only marginally to the development of GH-secreting adenomas (25). Recently, mutations affecting the gene encoding the catalytic subunit α of the PKA (*PRKACA*) have been reported in a large proportion of cortisol-secreting adrenocortical

adenomas (26, 27, 28, 29, 30), resulting in an increased PKA activity (31). Nevertheless, no hot spot mutations of *PRKACA* have been identified in a large cohort of GH-secreting adenomas (32). Finally, a recurrent somatic mutation in the *GPR101* gene, which encodes an orphan G-protein-coupled receptor (GPCR), has been recently reported in some adults with acromegaly (4% of cases) (33).

In addition, epidermal growth factor receptor (EGFR) overexpression has been described in hormonally active pituitary adenomas (34) and role for epidermal growth factor (EGF) and its receptor in the development and/or progression of pituitary tumors has been hypothesized (35). Dominant mutations in the deubiquitinase *USP8* gene that promote activation of EGFR signaling have been also found in adrenocorticotropin (ACTH)-secreting pituitary adenomas by exome sequencing (36). Finally, germline mutations of genes such as the aryl hydrocarbon receptor-interacting protein (*AIP*), the menin (*MEN1*), and the p27 (*CDKN1B*) have been reported in genetic syndromes associated with acromegaly (i.e., familial-isolated pituitary adenoma and multiple endocrine neoplasia type 1 and 4) and in a low percentage of young acromegalic patients (37).

Recently, Valimäki *et al.* investigated a small group of 12 patients with GH-secreting adenomas by whole-genome sequencing and single nucleotide polymorphism (SNP) array and did not find any novel recurrent genetic alteration (38). Aim of the present study was to perform a comprehensive genetic characterization of a large series of GH-secreting adenomas to identify novel genetic alterations potentially involved in tumorigenesis and/or in clinical outcome. To this aim, we used both targeted direct sequencing of *GNAS*, *PRKACA*, and *USP8* genes and next-generation exome sequencing.

Subjects and methods

Tissue samples, patients, and clinical annotations

Sporadic GH-secreting adenomas without familial or syndromic presentation were recruited in the present study. Accordingly, a total of 81 fresh frozen tumors were collected from four different participating European centers. If available, corresponding peripheral blood was also collected for the analysis. Inclusion criteria for participating in the study were a certified histological diagnosis of benign GH-secreting adenomas and available clinical data. The DNA was isolated as described previously (39). Qualitative and quantitative evaluation of the DNA was assessed by electrophoresis in a 1% agarose gel and

spectrophotometrically at 260 nm respectively. At the first screening, 14 tumor samples have been excluded due to insufficient DNA quality so that the final series included a total of 67 GH-secreting adenomas. Among them, the tumor samples were subdivided into two groups according to the availability of corresponding leukocyte DNA essential for next-generation whole-exome sequencing. Thus, 31 tumor samples underwent targeted direct sequencing for the analysis of selected genes (*GNAS*, *PRKACA*, and *USP8*) (Group 1), while the remaining 36 cases with corresponding leukocyte DNA were investigated by whole-exome sequencing (Group 2).

Clinical parameters, such as sex, age at diagnosis, date of surgery, tumor size, GH and IGF1 levels, presence of acromegaly-related complications, and follow-up data, were collected for all patients at the local centers. All the patients gave written informed consent, and the study was approved by the ethics committee at each participating institution.

Targeted and whole-exome sequencing and data analysis

For the targeted sequencing analysis, we focused on gene domains harboring alterations known or supposed biologically relevant in endocrine active tumors, i.e. known gain-of-function *GNAS* mutations (codon 201 and 227), mutations in the catalytic domain of the *PRKACA* (exon 7 and 8), and in the 14-3-3 binding domain and the microtubule-interacting and transport-domain (exons 1, 2, and 3) of the *USP8*, which is reported to be involved in regulating *USP8* catalytic function. The primers used for the targeted direct sequencing were generated with the Program Primer3Plus (39).

The complete list of the primers is reported in the [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article. In brief, PCR was performed on 1 µl of diluted DNA (2 ng/µl) in a final volume of 25 µl containing 1.5 mM MgCl₂, 0.2 µM of each primer, 200 µM dNTPs and 1 U *Taq* DNA polymerase. The reaction was started with an initial 95 °C denaturation step for 3 min, followed by 30 cycles of denaturation at 93 °C (20 s), annealing at 58 °C (30 s) and elongation at 72 °C (1 min). Direct sequencing of PCR products was performed using the QuickStart cycle sequencing kit (ABSciex Four Valley drive Concord, Ontario, Canada) on a CEQ8000 DNA analyzer (ABSciex).

For the next-generation sequencing, exomes were enriched in solution and indexed with the use of the SureSelect XT human all exon 50 Mb kit, version 5

(Agilent Technologies, Santa Clara, CA, USA). Sequencing was performed as paired-end reads of 100 bp on a HiSeq2500 systems (Illumina, San Diego, CA, USA) generating 8–14 Gb of sequence and an average depth of coverage between 110× and 170× on target regions. More than 95% of the target regions were covered 20 times or more. Pools of 12 indexed libraries were sequenced on four lanes. Image analysis and base calling were performed with the use of real-time analysis software (Illumina). Reads were aligned against the human assembly hg19 (GRCh37) using the Burrows-Wheeler Aligner tool (BWA v0.7.5a). Variant detection was done as described earlier (26).

Somatic variants have been evaluated by Polymorphism Phenotyping v2 tool (PolyPhen-2) (40) and scale invariant feature transform (SIFT) algorithm (<http://sift.jcvi.org/index.html>) (41). An unsupervised complete linkage clustering including the most relevant somatic mutations was performed by the Hamming distance as a similarity metric. The Gene Set Enrichment Analysis (GSEA) software was used for the gene enrichment and the functional annotation (Broad Institute, MSigDB database v5.0; <http://software.broadinstitute.org/gsea>) (42). A canonical pathway analysis (1330 gene set) and a gene family analysis were also performed with the same software.

Statistical analysis

Median, interquartile range, and frequency were used as descriptive statistics. IGF1 values were expressed as percentage of the upper limit of the normal range (%ULN). The Fisher's exact test or the χ^2 -test were used to investigate dichotomic variables, while a two-sided *t*-test (or non-parametric test) was used to test continuous variables. A non-parametric Kruskal-Wallis test, followed by the Bonferroni *post hoc* test, was used for multiple comparisons among several groups for non-normal distributed variables. Correlations and 95% CIs between the total number of mutations and different clinical parameters were evaluated by linear regression analysis. Statistical analyses were performed using the GraphPad Prism (version 5.0, La Jolla, CA, USA) and SPSS (PASW version 21.0, SPSS, Inc.) software. *P* values <0.05 were considered as statistically significant.

Results

Targeted DNA sequencing (Group 1)

A total of 31 patients affected by GH-secreting adenomas were included in this group. Minimum clinical data were

available for 17 of them (10 males and 7 females; median age: 46 years, range: 19–64; 16 macroadenomas and 1 microadenoma; median basal GH levels: 24.3 ng/ml, range: 2.3–333; median IGF1 %ULN: 391, range: 266–590).

We observed the presence of known activating *GNAS* mutations in five out of 31 evaluated samples (16.1% of cases), i.e. a p.Arg201Cys substitution in four samples and

a p.Gln227Leu in one sample. We did not identify any mutation in all the evaluated exons of *PRKACA* and *USP8* (Table 1). However, we detected different polymorphisms in the *USP8* gene: exon 1 (rs3131575 T/G heterozygous in eight cases and homozygous in one case, rs11632697 G/C heterozygous in 14 cases and homozygous in one case, and rs11632708 C/T heterozygous in 13 cases and homozygous

Table 1 List of genetic alterations at targeted sequencing of 31 GH-secreting pituitary adenomas. *PRKACA* exons 7 and 8 and *USP8* exons 2 and 3 showed no sequence variants and are not included.

Tumor ID (Group 1)	<i>GNAS</i>	<i>USP8</i> Ex 1	<i>USP8</i> 14-3-3 domain
1–16	WT	rs3131575 heterozygous	WT
1–18	WT	rs11632697 heterozygous	rs11638390 heterozygous
1–20	WT	WT	WT
1–24	WT	rs11632697 heterozygous, rs11632708 heterozygous	rs11638390 heterozygous
1–25	WT	rs11632697 heterozygous, rs11632708 heterozygous	rs11638390 heterozygous
1–26	WT	rs11632697 heterozygous, rs11632708 heterozygous	rs11638390 heterozygous
1–28	WT	WT	WT
1–29	WT	WT	WT
1–31	WT	WT	WT
1–32	WT	rs11632697 homozygous, rs11632708 homozygous	rs11638390 homozygous
1–33	WT	rs11632697 heterozygous, rs11632708 heterozygous, rs3131575 heterozygous	rs11638390 heterozygous
1–35	WT	WT	WT
1–37	WT	rs11632697 heterozygous, rs11632708 heterozygous, rs3131575 heterozygous	rs11638390 heterozygous
1–38	WT	WT	WT
1–39	WT	rs11632697 heterozygous, rs11632708 heterozygous	rs11638390 heterozygous
1–40	p.Q227L heterozygous	WT	WT
1–43	WT	WT	WT
1–44	WT	rs11632697 heterozygous, rs11632708 heterozygous	rs11638390 heterozygous
1–46	WT	rs3131575 heterozygous	WT
1–48	WT	WT	WT
1–50	p.R201C heterozygous	rs11632697 heterozygous, rs11632708 heterozygous	rs11638390 heterozygous
1–51	WT	rs11632697 heterozygous, rs11632708 heterozygous	rs11638390 heterozygous
1–52	WT	WT	rs11638390 heterozygous
1–53	p.R201C heterozygous	rs11632697 heterozygous, rs11632708 heterozygous	rs11638390 heterozygous
1–54	p.R201C heterozygous	WT	WT
1–55	rs121913494 heterozygous	rs11632697 heterozygous, rs11632708 heterozygous, rs3131575 heterozygous	rs11638390 heterozygous
1–57	WT	rs3131575 heterozygous	WT
1–58	p.R201C heterozygous	rs11632697 heterozygous, rs11632708 heterozygous, rs3131575 heterozygous	rs11638390 heterozygous
1–60	WT	rs3131575 homozygous	WT
1–61	WT	rs3131575 heterozygous	WT
1–62	WT	WT	WT

Bold values represent protein altering mutations.

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Table 2 Clinical and genetic data of the 36 patients affected by GH-secreting pituitary adenomas evaluated by next-generation exome-sequencing (Group 2).

	All	No mutations	Low number of mutation (≤ 3)	High number of mutations (> 3)	P
Clinical data at the time of diagnosis^a					
n	36	5	14	17	–
Sex (M/F)	16/18	1/4	6/6	8/9	NS
Age (years)	47.8 $\pm$ 19.0	32.4 $\pm$ 28.0	44.2 $\pm$ 7.0	54.1 $\pm$ 17.5	NS
Tumor size (mm)	18.8 $\pm$ 9.6	13.0 $\pm$ 3.5	20.6 $\pm$ 10.0	19.2 $\pm$ 11.2	NS
Micro/MACRO	2/32	1/4	1/11	1/16	NS
GH levels (μ g/l)					
Basal	24.3 $\pm$ 27.9	50.4 $\pm$ 58.5	29.7 $\pm$ 28.2	18.2 $\pm$ 14.4	NS
Post-OGTT nadir	21.6 $\pm$ 20.8	15.2 $\pm$ 16.0	29.7 $\pm$ 31.9	20.3 $\pm$ 18.9	NS
IGF1 levels (%UNL)	334 $\pm$ 152	312 $\pm$ 165	282 $\pm$ 122	372 $\pm$ 158	NS
Pituitary deficiencies (yes/no)	5/29	0/5	4/8	1/16	0.07
Co-secretion with prolactin (yes/no)	7/27	1/4	1/11	5/12	NS
Diabetes (yes/no)	10/22	1/4	3/9	6/11	NS
Hypertension (yes/no)	10/22	1/4	3/9	6/11	NS
Cardiac disease (yes/no)	3/29	0/5	1/11	2/15	NS
Clinical data after surgery					
Additional radiotherapy (yes/no)	3/27	0/5	0/12	3/12	NS
Additional drug therapy (yes/no)	9/19	3/2	4/8	2/9	NS
Biochemical remission (yes/no) ^b	9/7	1/3	5/2	3/4	NS
Genetic data					
Total number of mutations	175	0	1.6 $\pm$ 0.9	6.3 $\pm$ 3.1	<0.001
<i>GNAS</i> mutations (n – %)	11 (29.7%)	0	5 (35.7%)	6 (33%)	NS

M, male; F, female; micro, microadenoma; MACRO, macroadenoma; OGTT, oral glucose tolerance test; and %UNL, percentage of the upper normal limit.

^aThe clinical data from two patients are not available.

^bBiochemical remission evaluated 3–6 months after surgery.

in one case) and 14-3-3 binding domain (rs11638390 A/G heterozygous p.T739A in 14 cases and homozygous in one case) (Table 1). Allele frequencies did not differ significantly from frequencies reported in dbSNP database (<http://www.ncbi.nlm.nih.gov/SNP/>) (Supplementary Table 2, see section on supplementary data given at the end of this article).

Next-generation exome sequencing (Group 2)

The histopathological and clinical parameters for the patients included in this analysis are reported in the Table 2. At the whole-exome sequencing, we identified a total of 132 protein-altering somatic mutations in 36 samples, resulting in a median of three somatic mutations in exonic regions per sample (range: 0–13). The genetic alterations included 109 missense and seven nonsense mutations, 12 frameshift, two direct splicing, and two indel variations. According to the PolyPhen-2, 39 mutations were classified as probably damaging, 25 as possibly damaging, and 41 as benign. The entire list of the somatic

Figure 1

Total number of somatic mutations in the 36 GH-secreting pituitary tumors evaluated by next-generation exome sequencing (Group 2). The tumors affected by mutations in *GNAS* are represented with red bars. The numeration of the GH-secreting adenomas is consecutive and do not correspond to the tumor identification number.

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mutations including localization, gene symbols, and transcripts is reported in the [Supplementary Table 3](#), see section on [supplementary data](#) given at the end of this article.

We identified a subgroup of patients without any mutation (negative, $n=5$), a subgroup with a low number of mutations ($n=1-3$; $n=14$), and a subgroup with a high number of mutations ($n>3$; $n=17$) (Fig. 1). No significant correlation was observed between the total number of mutations and the evaluated clinical data, such as sex, age, tumor size and extension, and the initial GH levels.

The most frequent genetic alterations were the known gain-of-function mutations in the *GNAS* gene. Specifically, they were detected in 11 cases (31.4% of total, ten of them being females), encoding p.Arg201Cys substitution in seven samples, p.Arg201His in two samples, and p.Gln227Leu in two samples. No difference was observed in total number of mutations between the tumors with or without *GNAS* mutations (Fig. 1). No further genetic alterations were found in more than one sample in this series. Even comparing the list of the mutated genes with that of a recent paper on whole-genome sequencing in

12 GH-secreting adenomas (37), no additional recurrent somatic genetic alterations were observed.

However, some non-recurrent heterozygous somatic variants were observed in genes encoding GPCR, such as the chemokine receptor 10 (*CCR10*) and the olfactory receptor *OR51B4*, which are coupled to the Gs protein (activation of the cAMP signaling pathway), and the M3 muscarinic cholinergic receptor (*CHRM3*), which functions through Gq (activation of the inositol trisphosphate/calcium signaling pathway). Moreover, other non-recurrent alterations were found in genes coding for proteins involved in cAMP signaling pathway other than *GNAS*, such as the $\alpha 2$ catalytic subunit of the AMP-activated protein kinase (*PRKAA2*), the G-protein-coupled receptor kinase 3 (*GRK3*, alias *ADRBK2*), and the A1 subunit of the lysosomal H^+ ATPase (*ATP6V0A1*). Taken together, the mutations in genes involved in the cAMP signaling affected 14/36 samples (38.9% of total). Among them, nine samples presented only *GNAS* mutations, two samples mutations at *GNAS* and other genes of the cAMP signaling and three only mutations in other genes

Table 3 List of somatic mutations in genes associated with the cAMP signaling pathway or in the calcium signaling/metabolism detected in 36 GH-secreting pituitary adenomas (Group 2; the RefSeq annotation does not provide any annotated transcript for the position chr6:119532096, corresponding ENSEMBL identification 'ENST00000368466').

Gene symbol	Gene name	Base change	Protein change	Number of samples
cAMP signaling pathway				
<i>GNAS</i>	<i>GNAS</i> complex locus	c.601C>T c.602G>A c.584A>T	p.Arg201Cys p.Arg201His p. Gln227Leu	7 2 2
<i>PRKAA2</i>	Protein kinase, AMP-activated, $\alpha 2$ catalytic subunit	c.1132C>G	p.Pro378Ala	1
<i>ADRBK2</i>	Adrenergic, β , and receptor kinase 2	c.1976G>A	p.Arg659His	1
<i>ATP6V0A1</i>	ATPase, H^+ transporting, and lysosomal V0 subunit A1	c.94C>A	p.Leu32Ile	1
<i>CCR10</i>	Chemokine (C-C motif) receptor 10	c.691C>T	p.Gly231Ser	1
<i>CHRM3</i>	Cholinergic receptor and muscarinic 3	c.1244A>G	p.Asp415Gly	1
<i>OR51B4</i>	Olfactory receptor, family 51, subfamily B, and member 4	c.619C>T	p.Asp207Asn	1
Calcium signaling				
<i>CACNA1H</i>	Calcium channel, voltage-dependent, T type, and $\alpha 1H$ subunit	c.1175C>T	p.Ser392Leu	1
<i>CAPN1</i>	Calpain 1 and large subunit	c.380A>G	p.Asn127Ser	1
<i>DMD</i>	Dystrophin	c.960G>C	p.Ser320Arg	1
<i>GRIN2B</i>	Glutamate receptor, ionotropic and N-methyl D-aspartate 2B	c.1894C>A	p.Val632Leu	1
<i>JPH2</i>	Junctophilin 2	c.1111C>A	p.Glu371*	1
<i>MAN1A1</i>	Mannosidase, α , class 1A, and member 1	c.AG>>AA	NA	1
<i>PCDH11X</i>	Protocadherin 11 X-linked	c.3725C>G	p.Ala1242Gly	1
<i>PROCA1</i>	Protein interacting with cyclin A1	c.749C>G	p.Arg250Pro	1
<i>SLIT2</i>	Slit homolog 2	c.4190G>A	p.Cys139Tyr	1
<i>SPTA1</i>	Spectrin, α , erythrocytic 1	c.7201G>A	p.Arg2401*	1
<i>TESC</i>	Tescalcin	10_16delAGTGGGC, frameshift, 117537071		1

NA, not applicable.

*Corresponds to non-sense mutations according to the HVG5 recommendations.

Figure 2

Overview of the somatic mutations at genes involved in the cAMP signaling (i.e., CCR10, OR51B4, CHRM3, GNAS, PRKAA2, GRK3, and ATP6V0A1) or in the calcium signaling (i.e., CACNA1H, CAPN1, DMD, GRIN2B, JPH2, MAN1A1, PCDH11X, PROCA1, SLIT2, SPTA1, TESC) in GH-secreting adenomas evaluated by next-generation exome sequencing ($n=36$) and relationship with the total number of somatic mutations, sex, basal GH levels, and tumor extension at the time of diagnosis. Age: child < 18 years, young ≤ 50 years (median), old > 50 years; tumor size: macro = macroadenoma and micro = microadenoma; tumor extension: extra = extrasellar and intra = intrasellar; and GH: low ≤ 15 $\mu\text{g/l}$ (median) and high > 15 $\mu\text{g/l}$.

encoding GPCR or other members of the cAMP signaling. The corresponding details are reported in the Table 3.

Finally, a number of altered genes associated at different levels with the Ca^{2+} signaling and metabolism (i.e., involving both extra and intracellular compartment) were observed in eight cases (22.2% of total). They consisted in the $\alpha 1\text{H}$ subunit of the voltage-dependent T type calcium channel (*CACNA1H*), the large subunit of the calpain 1 (*CAPN1*), the dystrophin (*DMD*), the NMDA ionotropic glutamate receptor 2B (*GRIN2B*), the junctophilin 2 (*JPH2*), the mannosidase α class 1A (*MAN1A1*), the X-linked protocadherin 11 (*PCDH11X*), the protein interacting with cyclin A1 (*PROCA1*), the slit homolog 2 (*SLIT2*), the erythroid $\alpha 1$ spectrin (*SPTA1*), and the tescalcin (*TESC*) (Table 3).

An unsupervised clustering including all the somatic mutations in genes involved in the cAMP pathway or in the Ca^{2+} signaling was performed. The results including

the relationship with the total number of somatic mutations and clinical data are shown in the Fig. 2.

Concerning the correlation with the clinical data, the patients with mutations in genes of the cAMP signaling pathway were mostly females (10/14, 71%), while those with mutations in genes associated with the Ca^{2+} signaling were mostly males (5/7, 71%) and those with other kinds of mutations were equally distributed between the two sexes (50%, $P < 0.001$ by Kruskal–Wallis test for multiple comparisons) (Figs 2 and 3). A trend to a lower total number of mutations and younger age was observed in the group of patients without alterations of the cAMP or Ca^{2+} signaling in comparison with the other two groups (Figs 2 and 3). No significant differences in terms of tumor size and basal GH or IGF1 levels have been found.

Functional annotation and pathway analysis ▶ The gene enrichment analysis in the entire series identified a total of 117 altered genes associated with a gene ontology term. Several altered genes resulted to be associated with developmental biological processes (Supplementary Table 4, see section on supplementary data given at the end of this article). The canonical pathway analysis recognized no significant overlaps. The gene family analysis showed the presence of one cytokine/growth factor (*SLIT2*), seven protein kinases (*ADRBK2*, *CDK10*,

Figure 3

Relationship between the genetic alterations observed at the exome sequencing (i.e. mutations in genes member of the cAMP pathway, of the calcium signaling or in others) and clinical data (i.e. total number of somatic mutations in upper panel, age in the middle panel, and sex in the lower panel) in 36 evaluated GH-secreting adenomas.

CHUK, *EPHA8*, *PRKAA2*, *SCYL1*, and *TESK1*), four known oncogenes (*GNAS*, *KDMSA*, *SH3GL1*, and *STIL*) and two tumor suppressor genes (*SETD2* and *TSC2*) among the mutated genes.

Discussion

The present study offers a comprehensive genetic characterization of a large cohort of 67 GH-secreting pituitary adenomas. We aimed to identify novel molecular markers potentially involved in tumorigenesis and/or in clinical outcome. To this end, we first performed targeted sequencing of *GNAS*, *PRKACA*, and *USP8* genes in order to evaluate the presence of mutations in these genes in GH-secreting adenomas, finding only known *GNAS* gene mutations. By whole-exome sequencing, only a limited number of genetic alterations have been detected in the 36 evaluated samples. This finding is consistent with the low mitotic activity of pituitary tumors and with previous small studies on both non-functioning ($n=7$) (43) and GH-secreting pituitary adenomas ($n=12$) (37). Moreover, no recurrent somatic mutations have been observed, except the known alterations at the *GNAS* gene, similarly to a previous report on a small series of GH-secreting adenomas (37). In particular, no somatic mutations have been also detected at the gene *GPR101*, probably due to the low reported frequency of this mutations (11/248 cases) (33), and, at both the exome sequencing and the targeted sequencing, we did not find any mutations of the *PRKACA* and *USP8* genes. These findings further confirm that both these genetic alterations are not involved in the pathogenesis of GH-secreting adenomas (32, 36).

Interestingly, several non-recurrent alterations affected other genes involved in the cAMP signaling besides *GNAS* (see Table 2). These findings further support the view that deregulation of cAMP pathway is the most important pathogenetic mechanism in GH-secreting adenomas. Furthermore, a number of genes associated with the Ca^{2+} signaling (see Table 2) were altered. These findings are in agreement with another recent study on whole-genome alterations in 12 GH-secreting adenomas (37). This is consistent with the notion that binding of growth hormone-releasing hormone to its receptor activates not only the stimulatory subunit α of the G-protein ($\text{G}\alpha\text{-S}$, cAMP-dependent pathways) but also $\text{G}\alpha\text{-I}$, $\text{G}\beta$, and $\text{G}\gamma$, leading to release of intracellular free Ca^{2+} , which then further triggers secretion of GH (44, 45). Moreover, ATP, which is co-released with pituitary hormones, induces an increase in free Ca^{2+} in pituitary

cells (46). These data strongly suggest that dysregulation of the calcium signaling might be an important co-signal in somatotrophs and potentially involved in pituitary tumorigenesis. However, its biological role needs to be better investigated in future functional studies.

It has been suggested that tumors might be very heterogeneous with few mutations in common. Instead, different genes acting through the same molecular pathways may contribute to tumor formation (47). Therefore, it is possible that at least some of these low-frequency GH-secreting tumor variants present tumor-promoting mutations. Alternatively, they may present other types of molecular alterations not detectable by exome sequencing (i.e., mutations in non-coding intronic chromosomal regions).

In conclusion, we found no novel recurrently mutated genes in a large series of GH-secreting pituitary adenomas. However, our and previous genetic findings suggest that beside cAMP pathway, also different pathways, such as Ca^{2+} signaling, may play an important role in the pathogenesis of these tumors.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-15-1064>.

Declaration of interest

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

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Author contribution statement

C L Ronchi, B Allolio, M Reincke and M Fassnacht conceived the idea of the study. C L Ronchi developed the protocol of the study, coordinated the collection of the tissue materials and the clinical data, performed the statistical analysis, and wrote the first draft of the paper; E Peverelli, G Mantovani, and A Spada, provided the tumor tissue and the corresponding blood samples and contributed to write the paper; S Herterich performed the targeted sequencing analysis; I Weigand, D Calebiro, and S Sbiera contributed to the protocol of the study and to the data analysis; S Appenzeller performed the clustering and the heatmap; J Honegger, M Reincke, M Buchfelder, and J Flitsch provided the tumor tissue and the corresponding blood samples; T M Strom performed the whole-exome sequencing analysis including the filtering and the first data analysis; and M Fassnacht contributed to the coordination of the study and to write the paper. All the authors reviewed and approved the final version of the manuscript.

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References

- Chanson P, Salenave S, Kamenicky P, Cazabat L & Young J. Pituitary tumours: acromegaly. *Best Practice & Research* 2009 **23** 555–574. (doi:10.1016/j.beem.2009.05.010)
- Sherlock M, Ayuk J, Tomlinson JW, Toogood AA, Aragon-Alonso A, Sheppard MC, Bates AS & Stewart PM. Mortality in patients with pituitary disease. *Endocrine Reviews* 2010 **31** 301–342. (doi:10.1210/er.2009-0033)
- Melmed S, Kleinberg DL, Bonert V & Fleseriu M. Acromegaly: assessing the disorder and navigating therapeutic options for treatment. *Endocrine Practice* 2014 **20** (Suppl 1) 7–17 quiz 18–20. (doi:10.4158/EP14430.RA)
- Asa SL & Ezzat S. The pathogenesis of pituitary tumours. *Nature Reviews Cancer* 2002 **2** 836–849. (doi:10.1038/nrc926)
- Clayton RN & Farrell WE. Pituitary tumour clonality revisited. *Frontiers of Hormone Research* 2004 **32** 186–204.
- Vallar L, Spada A & Giannattasio G. Altered Gs and adenylate cyclase activity in human GH-secreting pituitary adenomas. *Nature* 1987 **330** 566–568. (doi:10.1038/330566a0)
- Landis CA, Masters SB, Spada A, Pace AM, Bourne HR & Vallar L. GTPase inhibiting mutations activate the alpha chain of Gs and stimulate adenylyl cyclase in human pituitary tumours. *Nature* 1989 **340** 692–696. (doi:10.1038/340692a0)
- Landis CA, Harsh G, Lyons J, Davis RL, McCormick F & Bourne HR. Clinical characteristics of acromegalic patients whose pituitary tumors contain mutant Gs protein. *Journal of Clinical Endocrinology and Metabolism* 1990 **71** 1416–1420. (doi:10.1210/jcem-71-6-1416)
- Clementi E, Malgaretti N, Meldolesi J & Taramelli R. A new constitutively activating mutation of the Gs protein alpha subunit-gsp oncogene is found in human pituitary tumours. *Oncogene* 1990 **5** 1059–1061.
- Lania AG, Mantovani G, Ferrero S, Pellegrini C, Bondioni S, Peverelli E, Braidotti P, Locatelli M, Zavanone ML, Ferrante E *et al.* Proliferation of transformed somatotroph cells related to low or absent expression of protein kinase a regulatory subunit 1A protein. *Cancer Research* 2004 **64** 9193–9198. (doi:10.1158/0008-5472.CAN-04-1847)
- Vitali E, Peverelli E, Giardino E, Locatelli M, Lasio GB, Beck-Peccoz P, Spada A, Lania AG & Mantovani G. Cyclic adenosine 3'-5'-monophosphate (cAMP) exerts proliferative and anti-proliferative effects in pituitary cells of different types by activating both cAMP-dependent protein kinase A (PKA) and exchange proteins directly activated by cAMP (Epac). *Molecular and Cellular Endocrinology* 2014 **383** 193–202. (doi:10.1016/j.mce.2013.12.006)
- Hayward BE, Barlier A, Korbonsits M, Grossman AB, Jacquet P, Enjalbert A & Bonthron DT. Imprinting of the G(s)alpha gene GNAS1 in the pathogenesis of acromegaly. *Journal of Clinical Investigation* 2001 **107** R31–R36. (doi:10.1172/JCI11887)
- Mantovani G, Ballare E, Giammona E, Beck-Peccoz P & Spada A. The gsalph gene: predominant maternal origin of transcription in human thyroid gland and gonads. *Journal of Clinical Endocrinology and Metabolism* 2002 **87** 4736–4740. (doi:10.1210/jc.2002-020183)
- Mantovani G, Bondioni S, Lania AG, Corbetta S, de Sanctis L, Cappa M, Di Battista E, Chanson P, Beck-Peccoz P & Spada A. Parental origin of Gsalph mutations in the McCune-Albright syndrome and in isolated endocrine tumors. *Journal of Clinical Endocrinology and Metabolism* 2004 **89** 3007–3009. (doi:10.1210/jc.2004-0194)
- Picard C, Silvy M, Gerard C, Buffat C, Lavaque E, Figarella-Branger D, Dufour H, Gabert J, Beckers A, Brue T *et al.* Gs alpha overexpression and loss of Gs alpha imprinting in human somatotroph adenomas: association with tumor size and response to pharmacologic treatment. *International Journal of Cancer* 2007 **121** 1245–1252. (doi:10.1002/ijc.22816)
- Petersenn S, Heyens M, Ludecke DK, Beil FU & Schulte HM. Absence of somatostatin receptor type 2 A mutations and gip oncogene in pituitary somatotroph adenomas. *Clinical Endocrinology* 2000 **52** 35–42. (doi:10.1046/j.1365-2265.2000.00880.x)
- Dong Q, Brucker-Davis F, Weintraub BD, Smallridge RC, Carr FE, Battey J, Spiegel AM & Shenker A. Screening of candidate oncogenes in human thyrotroph tumors: absence of activating mutations of the G alpha q, G alpha 11, G alpha s, or thyrotropin-releasing hormone receptor genes. *Journal of Clinical Endocrinology and Metabolism* 1996 **81** 1134–1140. (doi:10.1210/jcem.81.3.8772588)
- Farrell WE, Talbot JA, Bicknell EJ, Simpson D & Clayton RN. Genomic sequence analysis of a key residue (Arg183) in human G alpha q in invasive non-functional pituitary adenomas. *Clinical Endocrinology* 1997 **47** 241–244. (doi:10.1046/j.1365-2265.1997.2891088.x)
- Pack SD, Kirschner LS, Pak E, Zhuang Z, Carney JA & Stratakis CA. Genetic and histologic studies of somatomammotropic pituitary tumors in patients with the “complex of spotty skin pigmentation, myxomas, endocrine overactivity and schwannomas” (Carney complex). *Journal of Clinical Endocrinology and Metabolism* 2000 **85** 3860–3865. (doi:10.1210/jcem.85.10.6875)
- Kirschner LS, Carney JA, Pack SD, Taymans SE, Giatzakis C, Cho YS, Cho-Chung YS & Stratakis CA. Mutations of the gene encoding the protein kinase A type I-alpha regulatory subunit in patients with the Carney complex. *Nature Genetics* 2000 **26** 89–92. (doi:10.1038/79238)
- Yamasaki H, Mizusawa N, Nagahiro S, Yamada S, Sano T, Itakura M & Yoshimoto K. GH-secreting pituitary adenomas infrequently contain inactivating mutations of PRKAR1A and LOH of 17q23-24. *Clinical Endocrinology* 2003 **58** 464–470. (doi:10.1046/j.1365-2265.2003.01740.x)
- Sandrini F, Kirschner LS, Bei T, Farmakidis C, Yasufuku-Takano J, Takano K, Prezant TR, Marx SJ, Farrell WE, Clayton RN *et al.* PRKAR1A, one of the Carney complex genes, and its locus (17q22-24) are rarely altered in pituitary tumours outside the Carney complex. *Journal of Medical Genetics* 2002 **39** e78. (doi:10.1136/jmg.39.12.e78)
- Horvath A, Mathyakina L, Vong Q, Baxendale V, Pang AL, Chan WY & Stratakis CA. Serial analysis of gene expression in adrenocortical hyperplasia caused by a germline PRKAR1A mutation. *Journal of Clinical Endocrinology and Metabolism* 2006 **91** 584–596. (doi:10.1210/jc.2005-1301)
- Horvath A, Boikos S, Giatzakis C, Robinson-White A, Groussin L, Griffin KJ, Stein E, Levine E, Delimpasi G, Hsiao HP *et al.* A genome-wide scan identifies mutations in the gene encoding phosphodiesterase 11A4 (PDE11A) in individuals with adrenocortical hyperplasia. *Nature Genetics* 2006 **38** 794–800. (doi:10.1038/ng1809)
- Peverelli E, Ermetici F, Filopanti M, Elli FM, Ronchi CL, Mantovani G, Ferrero S, Bosari S, Beck-Peccoz P, Lania A *et al.* Analysis of genetic variants of phosphodiesterase 11A in acromegalic patients. *European Journal of Endocrinology* 2009 **161** 687–694. (doi:10.1530/EJE-09-0677)
- Beuschlein F, Fassnacht M, Assie G, Calebiro D, Stratakis CA, Osswald A, Ronchi CL, Wieland T, Sbiera S, Fauch FR *et al.* Constitutive activation of PKA catalytic subunit in adrenal Cushing's syndrome. *New England Journal of Medicine* 2014 **370** 1019–1028. (doi:10.1056/NEJMoa1310359)
- Cao Y, He M, Gao Z, Peng Y, Li Y, Li L, Zhou W, Li X, Zhong X, Lei Y *et al.* Activating hotspot L205R mutation in PRKACA and adrenal Cushing's syndrome. *Science* 2014 **344** 913–917. (doi:10.1126/science.1249480)
- Sato Y, Maekawa S, Ishii R, Sanada M, Morikawa T, Shiraishi Y, Yoshida K, Nagata Y, Sato-Otsubo A, Yoshizato T *et al.* Recurrent somatic mutations underlie corticotropin-independent Cushing's syndrome. *Science* 2014 **344** 917–920. (doi:10.1126/science.1252328)

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- 29 Goh G, Scholl UI, Healy JM, Choi M, Prasad ML, Nelson-Williams C, Kunstman JW, Korah R, Suttorp AC, Dietrich D *et al.* Recurrent activating mutation in PRKACA in cortisol-producing adrenal tumors. *Nature Genetics* 2014 **46** 613–617. (doi:10.1038/ng.2956)
- 30 Di Dalmazi G, Kisker C, Calebiro D, Mannelli M, Canu L, Arnaldi G, Quinkler M, Rayes N, Tabarin A, Laure Jullie M *et al.* Novel somatic mutations in the catalytic subunit of the protein kinase A as a cause of adrenal Cushing's syndrome: a European multicentric study. *Journal of Clinical Endocrinology and Metabolism* 2014 **99** E2093–E2100. (doi:10.1210/jc.2014-2152)
- 31 Calebiro D, Hannawacker A, Lyga S, Bathon K, Zabel U, Ronchi C, Beuschlein F, Reincke M, Lorenz K, Allolio B *et al.* PKA catalytic subunit mutations in adrenocortical Cushing's adenoma impair association with the regulatory subunit. *Nature Communications* 2014 **5** 5680. (doi:10.1038/ncomms6680)
- 32 Larkin SJ, Ferrau F, Karavitaki N, Hernandez-Ramirez LC, Ansorge O, Grossman AB & Korbonits M. Sequence analysis of the catalytic subunit of PKA in somatotroph adenomas. *European Journal of Endocrinology* 2014 **171** 705–710. (doi:10.1530/EJE-14-0545)
- 33 Trivellin G, Daly AF, Faucz FR, Yuan B, Rostomyan L, Larco DO, Schernthaner-Reiter MH, Szarek E, Leal LF, Caberg JH *et al.* Gigantism and acromegaly due to Xq26 microduplications and GPR101 mutation. *New England Journal of Medicine* 2014 **371** 2363–2374. (doi:10.1056/NEJMoa1408028)
- 34 LeRiche VK, Asa SL & Ezzat S. Epidermal growth factor and its receptor (EGF-R) in human pituitary adenomas: EGF-R correlates with tumor aggressiveness. *Journal of Clinical Endocrinology and Metabolism* 1996 **81** 656–662. (doi:10.1210/jcem.81.2.8636285)
- 35 Chaidarun SS, Eggo MC, Sheppard MC & Stewart PM. Expression of epidermal growth factor (EGF), its receptor, and related oncoprotein (erbB-2) in human pituitary tumors and response to EGF *in vitro*. *Endocrinology* 1994 **135** 2012–2021. (doi:10.1210/endo.135.5.7956924)
- 36 Reincke M, Sbiera S, Hayakawa A, Theodoropoulou M, Osswald A, Beuschlein F, Meitinger T, Mizuno-Yamasaki E, Kawaguchi K, Saeki Y *et al.* Mutations in the deubiquitinase gene USP8 cause Cushing's disease. *Nature Genetics* 2015 **47** 31–38. (doi:10.1038/ng.3166)
- 37 Valimaki N, Demir H, Pitkanen E, Kaasinen E, Karppinen A, Kivipelto L, Schalin-Jantti C, Aaltonen LA & Karhu A. Whole-genome sequencing of growth hormone (GH)-secreting pituitary adenomas. *Journal of Clinical Endocrinology and Metabolism* 2015 **100** 3918–3927. (doi:10.1210/jc.2015-3129)
- 38 Ronchi CL, Leich E, Sbiera S, Weismann D, Rosenwald A, Allolio B & Fassnacht M. Single nucleotide polymorphism microarray analysis in cortisol-secreting adrenocortical adenomas identifies new candidate genes and pathways. *Neoplasia* 2012 **14** 206–218. (doi:10.1593/neo.111758)
- 39 Untergasser A, Nijveen H, Rao X, Bisseling T, Geurts R & Leunissen JA. Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Research* 2007 **35** W71–W74. (doi:10.1093/nar/gkm306)
- 40 Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS & Sunyaev SR. A method and server for predicting damaging missense mutations. *Nature Methods* 2010 **7** 248–249. (doi:10.1038/nmeth0410-248)
- 41 Kumar P, Henikoff S & Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nature Protocols* 2009 **4** 1073–1081. (doi:10.1038/nprot.2009.86)
- 42 Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES *et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *PNAS* 2005 **102** 15545–15550. (doi:10.1073/pnas.0506580102)
- 43 Newey PJ, Nesbit MA, Rimmer AJ, Head RA, Gorvin CM, Attar M, Gregory L, Wass JA, Buck D, Karavitaki N *et al.* Whole-exome sequencing studies of nonfunctioning pituitary adenomas. *Journal of Clinical Endocrinology and Metabolism* 2013 **98** E796–E800. (doi:10.1210/jc.2012-4028)
- 44 Meier K, Knebel W & Schofl C. Potassium depolarization elevates cytosolic free calcium concentration in rat anterior pituitary cells through 1,4-dihydropyridine-sensitive, omega-conotoxin-insensitive calcium channels. *Endocrinology* 1988 **122** 2764–2770. (doi:10.1210/endo-122-6-2764)
- 45 Canny BJ, Rawlings SR & Leong DA. Pituitary adenylate cyclase-activating polypeptide specifically increases cytosolic calcium ion concentration in rat gonadotropes and somatotropes. *Endocrinology* 1992 **130** 211–215. (doi:10.1210/endo.130.1.1727697)
- 46 Villalobos C, Alonso-Torre SR, Nunez L & Garcia-Sancho J. Functional ATP receptors in rat anterior pituitary cells. *American Journal of Physiology* 1997 **273** C1963–C1971.
- 47 Wood LD, Parsons DW, Jones S, Lin J, Sjoblom T, Leary RJ, Shen D, Boca SM, Barber T, Ptak J *et al.* The genomic landscapes of human breast and colorectal cancers. *Science* 2007 **318** 1108–1113. (doi:10.1126/science.1145720)

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