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Landscape of somatic mutations in sporadic GH-secreting pituitary adenomas

¹Cristina L. Ronchi, ²Erika Peverelli, ³Sabine Herterich, ¹Isabel Weigand, ²Giovanna Mantovani,
⁴Thomas Schwarzmayr, ⁵Silviu Sbiera, ¹Bruno Allolio, ⁶Jürgen Honegger, ^{5,7}Silke Appenzeller,
⁸Andrea G Lania, ⁶Martin Reincke, ⁹Davide Calebiro, ²Anna Spada, ¹⁰Michael Buchfelder,
¹¹Joerg Flitsch, ^{4,12}Tim M. Strom, ^{1,5}Martin Fassnacht.

¹Department of Internal Medicine I, Endocrine and Diabetes Unit, University Hospital, University of
Wuerzburg, Wuerzburg, Germany; ²Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico,
Endocrinology and Diabetology Unit, University of Milan, Dpt. of Clinical sciences and Community
Health; Milan, Italy; ³Central Laboratory, University Hospital, University of Wuerzburg, Wuerzburg,
Germany; ⁴Institute of Human Genetics, Helmholtz Zentrum Munich, Neuherberg, Germany;
⁵Comprehensive Cancer Center Mainfranken, University of Wuerzburg, Germany; ⁶Medizinische
Klinik and Poliklinik IV, Ludwig-Maximilians University, Munich, Germany; ⁷Core Unit Systems
Medicine, University of Wuerzburg, Wuerzburg, Germany, ⁸Department of Biomedical Sciences,
Humanitas University, Endocrinology Unit, Humanitas Research Hospital, Rozzano, Milan, Italy;
⁹University of Wuerzburg, Institute of Pharmacology and Toxicology and Bioimaging Center,
Wuerzburg, Germany; ¹⁰Department of Neurosurgery, University Hospital of Erlangen, Germany;
¹¹Neurosurgery, University Hospital of Hamburg-Eppendorf, Hamburg, Germany; ¹²Institute of
Human Genetics, Technische Universitaet Muenchen, Munich, Germany.

Short title: genetic pattern of GH-secreting adenomas

Key words: acromegaly, pathogenesis, molecular alterations

Correspondence:

Cristina L Ronchi, MD, PhD
Endocrine and Diabetes Unit
University Hospital of Wuerzburg
Oberduerrbacherstr. 6
97080 Wuerzburg, Germany
Ronchi_C@ukw.de

Abstract

Context: Alterations in the cAMP signalling 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 5 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 3 mutations per sample (range: 1-13). The only recurrent mutations have been observed in *GNAS* (31.4% of cases). However, 7 genes involved in cAMP signalling pathway were affected in 14 of 36 samples and 8 samples harbored variants in genes involved in the calcium signalling or metabolism. At the enrichment analysis, several altered genes resulted to be associated with developmental processes. No correlation between genetic alterations and the clinical data was observed.

Conclusions: 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 signalling might be involved in the pathogenesis of these tumors.

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61 **Introduction**

62 Pituitary tumors represent approximately 15% of all primary intracranial lesions. Growth hormone
63 (GH)-secreting pituitary adenomas are the second most frequent type of hormone-producing pituitary
64 tumors, after prolactin-secreting adenomas¹. Excessive secretion of GH causes gigantism during
65 childhood and acromegaly in adults, with significant morbidity due to clinical complications involving
66 cardiovascular, respiratory, and metabolic systems.^{2,3}

67 The monoclonal origin of most pituitary adenomas indicates that these tumors derive from the
68 replication of a single cell that acquired growth advantage. The latter has been suggested to result from
69 genetic or epigenetic alterations leading to activation of proto-oncogenes or inactivation of tumor
70 suppressor genes^{4,5}. However, despite intensive investigations, little is known about the genetic causes
71 of pituitary adenomas. The only mutations identified to date in a significant proportion (30-40%) of
72 sporadic GH-secreting adenomas occur in the gene encoding the α subunit of the stimulatory G protein
73 (*GNAS*).⁶⁻⁹ These somatic activating mutations (*gsp* mutations), found in codon 201 and 227, prevent
74 hydrolysis of GTP, leading to a constitutive activation of the cAMP pathway, which in somatotrophs
75 and in other endocrine cells acts as a mitogenic signal^{10,11}. In somatotrophs the *GNAS* transcript is
76 expressed mainly from the maternal allele, due to tissue-specific paternal imprinting^{12,13}. Consistently,
77 *gsp* mutations in sporadic GH-secreting adenomas are found on the maternal allele¹⁴ and partial loss of
78 this imprinting is present in tumors negative for *gsp* mutations¹⁵, further supporting the involvement of
79 *GNAS* locus in pituitary tumorigenesis. So far, the screening for mutations in other G-protein subunits
80 in pituitary tumors has given negative results.¹⁶⁻¹⁸

81 Genetic alterations in other genes involved in cAMP signalling have been identified as cause
82 of other endocrine tumors. A reduced expression and/or function of the PKA regulatory subunit type
83 I α (*PRKAR1A*) due to loss-of-function mutations, leading to an abnormal cAMP pathway activation,
84 causes GH-secreting pituitary adenomas in Carney complex, an autosomal dominant familial
85 syndrome^{19,20}. To date, mutations of *PRKAR1A* gene have been rarely found in sporadic pituitary
86 tumors^{21,22}, although a reduced *PRKAR1A* expression resulting from increased proteosomal
87 degradation has been described in sporadic GH-secreting tumors¹⁰. Reduced cAMP degradation
88 caused by mutations in *PDE11A* and *PDE8B*, coding for members of the phosphodiesterase (PDE)

89 family, have been involved in adrenocortical hyperplasia, adenomas and cancer as well as in testicular
90 germ cell tumors^{23, 24}. However, genetic variants of *PDE11A4* contribute only marginally to the
91 development of GH-secreting adenomas²⁵. Recently, mutations affecting the gene encoding the
92 catalytic subunit α of the PKA (*PRKACA*) have been reported in a large proportion of cortisol-
93 secreting adrenocortical adenomas²⁶⁻³⁰, resulting in increased PKA activity³¹. Nevertheless, no hot spot
94 mutations of *PRKACA* have been identified in a large cohort of GH-secreting adenomas³². Finally, a
95 **recurrent somatic mutation** in the *GPR101* gene, which encodes an orphan G-protein-coupled
96 receptor, has been recently reported in some adults with acromegaly (4% of cases).³³

97 In addition, epidermal growth factor receptor (EGFR) overexpression has been described in
98 hormonally active pituitary adenomas³⁴ and role for EGF and its receptor in the development and/or
99 progression of pituitary tumors has been hypothesized³⁵. Dominant mutations in the deubiquitinase
100 *USP8* gene that promote activation of EGFR signalling have been also found in ACTH-secreting
101 pituitary adenomas by exome sequencing³⁶. Finally, germline mutations of genes such as the aryl
102 hydrocarbon receptor interacting protein (*AIP*), the menin (*MEN1*) and the p27 (*CDKN1B*) have been
103 reported in genetic syndromes associated with acromegaly (i.e. familial isolated pituitary adenoma and
104 multiple endocrine neoplasia type 1 and 4) and in a low percentage of young acromegalic patients.³⁷

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

111

112 **Material and methods**

113 *Tissue samples, patients and clinical annotations*

114 **Sporadic GH-secreting adenomas without familial or syndromic presentation were recruited in the**
115 **present study.** Accordingly, a total of 81 fresh frozen tumors were collected from 4 different
116 participating European centers. If available, corresponding peripheral blood was also collected for the

117 analysis. Inclusion criteria for participating in the study were a certified histological diagnosis of
118 benign GH-secreting adenomas and available clinical data. The DNA was isolated as described
119 previously.³⁹ Qualitative and quantitative evaluation of the DNA was assessed by electrophoresis in a
120 1% agarose gel and spectro-photometrically at 260 nm, respectively. At the first screening, 14 tumor
121 samples have been excluded due to insufficient DNA quality, so that the final series included a total of
122 67 GH-secreting adenomas. Among them, the tumor samples were subdivided into two groups
123 according to the availability of corresponding leukocyte-DNA essential for next generation whole
124 exome-sequencing. Thus, 31 tumor samples underwent targeted direct sequencing for the analysis of
125 selected genes (*GNAS*, *PRKACA* and *USP8*) (Group 1), while the remaining 36 cases with
126 corresponding leukocyte-DNA were investigated by whole exome-sequencing (Group 2).

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

131

132 *Targeted and whole-exome sequencing and data analysis*

133 For the targeted sequencing analysis we focused on gene domains harboring alterations known or
134 supposed biologically relevant in endocrine active tumors, i.e. known gain-of-function *GNAS*
135 mutations (codon 201 and 227), mutations in the catalytic domain of the *PRKACA* (exon 7 and 8) and
136 in the 14-3-3- binding domain and the MIT (Microtubule Interacting and Transport)-domain (exons 1,
137 2, 3) of the *USP8*, which is reported to be involved in regulating *USP8* catalytic function. In addition,
138 we also evaluated the presence of the known hot spot *GNAS* mutations. The primers used for the
139 targeted direct sequencing were generated with the Program Primer3Plus³⁹.

140 The complete list of the primers is reported in the *Supplementary Table 1*. In brief, PCR was
141 performed on 1 µl of diluted DNA (2 ng/µl) in a final volume of 25 µl containing 1,5 mM MgCl₂, 0,2
142 µM of each primer, 200 µM dNTPs and 1 U *Taq* DNA Polymerase. The reaction was started with an
143 initial 95 °C denaturation step for 3 min, followed by 30 cycles of denaturation at 93 °C (20 sec),
144 annealing at 58 °C (30 sec) and elongation at 72 °C (1 min). Direct sequencing of PCR products was

145 performed using the QuickStart Cycle Sequencing Kit (ABSciex) on a CEQ8000 DNA Analyzer
146 (ABSciex).

147 For the next generation sequencing, exomes were enriched in solution and indexed with the
148 use of the SureSelect XT Human All Exon 50Mb kit, version 5 (Agilent Technologies, Santa Clara,
149 CA, USA). Sequencing was performed as paired-end reads of 100bp on a HiSeq2500 systems
150 (Illumina, S. Diego, CA, USA) generating 8-14 Gb of sequence and an average depth of coverage
151 between 110x and 170x on target regions. More than 95% of the target regions were covered 20 times
152 or more. Pools of 12 indexed libraries were sequenced on four lanes. Image analysis and base calling
153 were performed with the use of Real-Time Analysis software (Illumina). Reads were aligned against
154 the human assembly hg19 (GRCh37) using Burrows-Wheeler Aligner (BWA v 0.7.5a). Variant
155 detection was done as described earlier.²⁶

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

162

163 *Statistical analysis*

164 Median, interquartile range (IQR), and frequency were used as descriptive statistics. IGF-I values were
165 expressed as percentage of the upper limit of the normal range (%ULN). The Fisher's exact test or the
166 Chi-square test were used to investigate dichotomic variables, while a two-sided *t* test (or non-
167 parametric test) was used to test continuous variables. A non-parametric Kruskal-Wallis test, followed
168 by the Bonferroni *post-hoc* test, was used for multiple comparisons among several groups for non-
169 normal distributed variables. Correlations and 95% confidence intervals (95%CI) between the total
170 number of mutations and different clinical parameters were evaluated by linear regression analysis.
171 Statistical analyses were performed using the GraphPad Prism (version 5.0, La Jolla, CA, USA) and

172 SPSS (PASW Version 21.0, SPSS Inc., Chicago, IL, USA) software. P values <0.05 were considered
173 as statistically significant.

174

175 **Results**

176

177 *Targeted DNA sequencing (Group 1)*

178 A total of 31 patients affected by GH-secreting adenomas were included in this group. Minimum
179 clinical data were available for 17 of them (10M&7F; median age: 46 yrs, range: 19-64; 16
180 macroadenomas and 1 microadenoma; median basal GH levels: 24.3 ng/mL, range: 2.3-333; median
181 IGFI %ULN: 391, range: 266-590).

182 We observed the presence of known activating *GNAS* mutations in 5 out of 31 evaluated samples
183 (16.1% of cases), i.e. a p.Arg201Cys substitution in 4 samples and a p.Gln227Leu in 1 sample. We did
184 not identify any mutation in all the evaluated exons of *PRKACA* and *USP8* (**Table 1**). However, we
185 detected different polymorphisms in the *USP8* gene: exon 1 (rs3131575 T/G heterozygous in 8 cases
186 and homozygous in 1 case, rs11632697 G/C heterozygous in 14 cases and homozygous in 1 case,
187 rs11632708 C/T heterozygous in 13 cases and homozygous in 1 case) and 14-3-3 binding domain
188 (rs11638390 A/G heterozygous p.T739A in 14 cases and homozygous in 1 case) (**Table 1**). Allele
189 frequencies did not differ significantly from frequencies reported in dbSNP database
190 (<http://www.ncbi.nlm.nih.gov/SNP/>) (**Supplementary Table 2**).

191

192 *Next-generation exome-sequencing (Group 2)*

193

194 The histopathological and clinical parameters for the patients included in this analysis are reported in
195 the **Table 2**. At the whole-exome sequencing, we identified a total of 132 protein-altering somatic
196 mutations in 36 samples, resulting in a median of 3 somatic mutations in exonic regions per sample
197 (range: 0-13). The genetic alterations included 109 missense and 7 nonsense mutations, 12 frameshift,
198 2 direct splicing and 2 indel variations. According to the PolyPhen-2, 39 mutations were classified as
199 probably damaging, 25 as possibly damaging, and 41 as benign. The entire list of the somatic

200 mutations including localization, gene symbols and transcripts is reported in the *Supplementary Table*
201 **3**.

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

206 The most frequent genetic alterations were the known gain-of-function mutations in the *GNAS*
207 gene. Specifically, they were detected in 11 cases (31.4% of total, 10 of them being females),
208 encoding p.Arg201Cys substitution in 7 samples, p.Arg201His in 2 samples and p.Gln227Leu in 2
209 samples. No difference was observed in total number of mutations between the tumors with or without
210 *GNAS* mutations (**Figure 1**). No further genetic alterations were found in more than one sample in this
211 series. Even comparing the list of the mutated genes with that of a recent paper on whole-genome
212 sequencing in 12 GH-secreting adenomas³⁷, no additional recurrent somatic genetic alterations were
213 observed.

214 However, some non-recurrent heterozygous somatic variants were observed in genes encoding
215 G-protein coupled receptors (GPCR), such as the chemokine receptor 10 (*CCR10*) and the olfactory
216 receptor *OR51B4*, which are coupled to the Gs protein (activation of the cAMP signalling pathway),
217 and the M3 muscarinic cholinergic receptor (*CHRM3*), which functions through Gq (activation of the
218 inositol trisphosphate/calcium signalling pathway). Moreover, other non-recurrent alterations were
219 found in genes coding for proteins involved in cAMP signalling pathway other than *GNAS*, such as the
220 $\alpha 2$ catalytic subunit of the AMP-activated protein kinase (*PRKAA2*), the G-protein-coupled receptor
221 kinase 3 (*GRK3*, alias *ADRBK2*), the A1 subunit of the lysosomal H⁺ ATPase (*ATP6V0A1*). Taken
222 together, the mutations in genes involved in the cAMP signalling affected 14/36 samples (38.9% of
223 total). Among them, 9 samples presented only *GNAS* mutations, 2 samples mutations at *GNAS* and
224 other genes of the cAMP signalling and 3 only mutations in other genes encoding GPCR or other
225 members of the cAMP signalling. The corresponding details are reported in the **Table 3**.

226 Finally, a number of altered genes associated at different levels with the Ca²⁺ signalling and
227 metabolism (i.e. involving both extra- and intracellular compartment) were observed in 8 cases (22.2%

228 of total). They consisted in the $\alpha 1H$ subunit of the voltage-dependent T type calcium channel
229 (*CACNA1H*), the large subunit of the calpain 1 (*CAPN1*), the dystrophin (*DMD*), the NMDA
230 ionotropic glutamate receptor 2B (*GRIN2B*), the junctophilin 2 (*JPH2*), the mannosidase α class 1A
231 (*MAN1A1*), the X-linked protocadherin 11 (*PCDH11X*), the protein interacting with cyclin A1
232 (*PROCA1*), the slit homolog 2 (*SLIT2*), the erythroid $\alpha 1$ spectrin (*SPTA1*), and the tescalcin (*TESC*)
233 (**Table 3**).

234 An unsupervised clustering including all the somatic mutations in genes involved in the cAMP
235 pathway or in the Ca^{2+} signalling was performed. The results, including the relationship with the total
236 number of somatic mutations and clinical data is shown in the **Figure 2**.

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

245 *Functional annotation and pathway analysis:* The gene enrichment analysis in the entire series
246 identified a total of 117 altered genes associated with a gene ontology (GO) term. Several altered
247 genes resulted to be associated with developmental biological processes (**Supplementary table 4**). The
248 canonical pathway analysis recognized no significant overlaps. The gene family analysis showed the
249 presence of 1 cytokine/growth factor (*SLIT2*), 7 protein kinases (*ADRBK2*, *CDK10*, *CHUK*, *EPHA8*,
250 *PRKAA2*, *SCYLI*, *TESK1*), 4 known oncogenes (*GNAS*, *KDM5A*, *SH3GL1*, *STIL*) and 2 tumor
251 suppressor genes (*SETD2*, *TSC2*) among the mutated genes.

252

253 Discussion

254 The present study offers a comprehensive genetic characterization of a large cohort of 67 GH-
255 secreting pituitary adenomas. We aimed to identify novel molecular markers potentially involved in

256 tumorigenesis and/or in clinical outcome. To this end, we first performed targeted sequencing of
257 *GNAS*, *PRKACA* and *USP8* genes in order to evaluate the presence of mutations in these genes in GH-
258 secreting adenomas, finding only known *GNAS* gene mutations. By whole-exome sequencing, only a
259 limited number of genetic alterations have been detected in the 36 evaluated samples. This finding is
260 consistent with the low mitotic activity of pituitary tumors and with previous small studies on both
261 non-functioning (n=7)⁴³ and GH-secreting pituitary adenomas (n=12)³⁷. Moreover, no recurrent
262 somatic mutations have been observed, except the known alterations at the *GNAS* gene, similarly to a
263 previous report on a small series of GH-secreting adenomas³⁷. In particular, no somatic mutations have
264 been also detected at the gene *GPR101*, probably due to the low reported frequency of this mutations
265 (11/248 cases)³³, and, at both the exome-sequencing and the targeted sequencing, we did not find any
266 mutations of the *PRKACA* and *USP8* genes. These findings further confirm that both these genetic
267 alterations are not involved in the pathogenesis of GH-secreting adenomas^{32, 36}.

268 Interestingly, several non-recurrent alterations affected other genes involved in the cAMP
269 signalling besides *GNAS* (see Table 2). These findings further support the view that deregulation of
270 cAMP pathway is the most important pathogenetic mechanism in GH-secreting adenomas.
271 Furthermore, a number of genes associated with the Ca²⁺ signalling (see Table 2) were altered. These
272 findings are in agreement with another recent study on whole-genome alterations in 12 GH-secreting
273 adenomas³⁷. This is consistent with the notion that binding of GHRH to its receptor activates not only
274 the stimulatory subunit α of the G-protein (G α -S, cAMP-dependent pathways), but also G α -I, G β and
275 G γ leading to release of intracellular free Ca²⁺, which then further triggers secretion of GH^{44, 45}.
276 Moreover, ATP, which is co-released with pituitary hormones, induces an increase in free Ca²⁺ in
277 pituitary cells⁴⁶. These data strongly suggest that dysregulation of the calcium signalling might be an
278 important co-signal in somatotrops and potentially involved in pituitary tumorigenesis. **However, its**
279 **biological role needs to be better investigated in future functional studies.**

280 It has been suggested that tumors might be very heterogeneous with few mutations in
281 common. Instead, different genes acting through the same molecular pathways may contribute to
282 tumor formation⁴⁷. Therefore, it is possible that at least some of these low-frequency GH-secreting
283 tumor variants present tumor promoting mutations. Alternatively, they may present other types of

284 molecular alterations not detectable by exome-sequencing (i.e. mutations in non-coding intronic
285 chromosomal regions).

286 In conclusion, we found no novel recurrently mutated genes in a large series of GH-secreting
287 pituitary adenomas. However, our and previous genetic findings suggest that beside cAMP pathway,
288 also different pathways, such as Ca²⁺ signalling, may play an important role in the pathogenesis of
289 these tumors.

290

291 **Declaration of interest**

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

294

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299

300 **Author contribution**

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

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314

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470 **Legend to the figures**

471

472 **Figure 1.** Total number of somatic mutations in the 36 GH-secreting pituitary tumors evaluated by
473 next-generation exome-sequencing (Group 2). The tumors affected by mutations in *GNAS* are
474 represented with red bars. The numeration of the GH secreting adenomas is consecutive and do not
475 correspond to the tumor identification number.

476

477 **Figure 2.** Overview of the somatic mutations at genes involved in the cAMP signalling (i.e. *CCR10*,
478 *OR51B4*, *CHRM3*, *GNAS*, *PRKAA2*, *GRK3*, *ATP6V0A1*) or in the calcium signalling (i.e. *CACNA1H*,
479 *CAPN1*, *DMD*, *GRIN2B*, *JPH2*, *MAN1A1*, *PCDH11X*, *PROCA1*, *SLIT2*, *SPTA1*, *TESC*) in GH-
480 secreting adenomas evaluated by next-generation exome-sequencing (n=36) and relationship with the
481 total number of somatic mutations, sex, basal GH levels, and tumor extension at the time of diagnosis.
482 Age: child < 18 years, young \leq 50 years (median), old >50 years; tumor size: macro=macroadenoma,
483 micro=microadenoma; tumor extension: extra=extrasellar, intra=intrasellar; GH: low \leq 15 μ g/l
484 (median), high >15 μ g/l.

485

486 **Figure 3.** Relationship between the genetic alterations observed at the exome-sequencing (i.e.
487 mutations in genes member of the cAMP pathway, of the calcium signalling or in others) and clinical
488 data (i.e. total number of somatic mutations in upper panel, age in the middle panel; sex in the lower
489 panel) in 36 evaluated GH-secreting adenomas.

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491

492 **Suppl Table 3.** List of all genetic alterations observed in 36 GH-secreting pituitary tumors by next-
493 generation exome-sequencing (Group 2), including gene symbols, transcripts and aminoacid
494 substitutions.