Genetic and Epigenetic Characterization of Growth Hormone–Secreting Pituitary Tumors

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Abstract

Somatic driver mechanisms of pituitary adenoma pathogenesis have remained incompletely characterized; apart from mutations in the stimulatory Gz protein (Gz, encoded by GNAS) causing activated cAMP synthesis, pathogenic variants are rarely found in growth hormone–secreting pituitary tumors (somatotropinomas). The purpose of the current work was to clarify how genetic and epigenetic alterations contribute to the development of somatotropinomas by conducting an integrated copy number alteration, whole-genome and bisulfit sequencing, and transcriptome analysis of 21 tumors. Somatic mutation burden was low, but somatotropinomas formed two subtypes associated with distinct aneuploidy rates and unique transcription profiles. Tumors with recurrent chromosome aneuploidy (CA) were GNAS mutation negative (Gsp−). The chromosome stable (CS)–group contained Gsp+ somatotropinomas and two totally aneuploidy-free Gsp− tumors. Genes related to the mitotic G1–S-checkpoint transition were differentially expressed in CA- and CS-tumors, indicating difference in mitotic progression. Also, pituitary tumor transforming gene 1 (PTTG1), a regulator of sister chromatid segregation, showed abundant expression in CA-tumors. Moreover, somatotropinomas displayed distinct Gsp genotype–specific methylation profiles and expression quantitative methylation (eQTM) analysis revealed that inhibitory G Di signaling is activated in Gsp+ tumors. These findings suggest that aneuploidy through modulated driver pathways may be a causative mechanism for tumorigenesis in Gsp+ somatotropinomas, whereas Gsp− tumors with constitutively activated cAMP synthesis seem to be characterized by DNA methylation activated Gz, signaling.

Implications: These findings provide valuable new information about subtype-specific pituitary tumorigenesis and may help to elucidate the mechanisms of aneuploidy also in other tumor types.

Introduction

Pituitary adenomas are common and comprise 15% of all diagnosed intracranial neoplasms. The overall rate of pituitary tumors in the general population is one case in 1,064 (1). The most common functioning pituitary tumors hypersecrete prolactin (PRL; 40%). Growth hormone (GH)–secreting adenomas (somatotropinomas) constitute 15% to 20% and usually lead to increased height (gigantism) in children or adolescents. In adults, hypersecretion of GH causes acromegaly and leads to overgrowth of bone and cartilage, insulin resistance, hypertension, cardiovascular and respiratory complications, and increased risk of neoplasms. Despite being benign, excess GH production is associated with increased morbidity and reduced life expectancy (2–4).

The majority of pituitary adenomas arise in a sporadic setting and are considered to be unicellular in origin. As in other neoplasms, pituitary tumor formation and dysregulated hormone secretion are results of series of genetic and epigenetic alterations upsetting the balance between proliferation and apoptosis. The most frequently described somatic pathogenic events occurring in somatotropinomas are gain-of-function mutations in the stimulatory guanine nucleotide (GTP) binding protein alpha (Gzx) encoded by the GNAS gene. This Gsp oncogene contributes to constitutive synthesis of cyclic adenosine monophosphate (cAMP), activation of protein kinase A (PKA) pathway, and subsequent tumor formation. Gsp mutations occur in approximately 35% of GH-producing pituitary adenomas (5, 6). Next-generation sequencing has shown that the somatic background of pituitary adenomas is calm and single nucleotide– (SNV) and small structural variants (SV) are rarely found. Therefore, the exact mechanisms of tumorigenesis often remain unknown (7–12).

Numerical alterations of whole chromosomes, aneuploidy, is observed in a subset of pituitary tumors (8, 12). Aneuploidy is frequently noted in solid and malignant tumors and is associated with tumor recurrence and drug resistance in some tumor types (13, 14). Shuffling of genomic content through aneuploidy facilitates loss of heterozygosity (LOH) of tumor suppressors and increases copy number of oncogenes and can constitute a powerful driver for tumor progression. In addition, epigenetic modifications associated to changes in gene expression are
considered potential causes of pituitary tumor initiation and development (15).

Apart from the Gsp+ driver mutation, mechanisms of pituitary adenoma pathogenesis have remained incompletely characterized, and improved understanding of uncontrolled cell growth associated with pituitary tumors is required. The purpose of the current work was to clarify how somatic alterations drive development of somatotropinomas and discover subgroup-specific somatic patterns. This was done by dissecting associations between somatic copy number alterations (SCNA), gene expression, and DNA methylation in GNAS mutation negative (Gsp+) and positive (Gsp+) pituitary tumors. In addition, whole-genome sequencing (WGS) was performed to identify somatic SNV and SV changes.

Here, we found that somatotropinomas form two tumor subtypes associated with distinct aneuploidy rates and transcriptional profiles. Our results provide evidence that defective chromosomal segregation may underlie the development of aneuploidy and tumor initiation in a subset of Gsp− somatotropinomas. Furthermore, we show that Gsp mutation status is the major determinant of methylation profiles of somatotropinomas, and that methylation regulated transcription seems to activate an adaptive response to elevated cAMP levels in Gsp+ tumors.

Materials and Methods

Patient material

We studied somatotropinomas from 21 patients [13 males and 8 females, mean age at diagnosis 43 years (range, 14–69 years); Table 1]. The tumor samples were collected between 2009 and 2015 at the Helsinki University Hospital and frozen while fresh. The study was approved by the Ethics Committee of the Hospital District of Helsinki (Dnr. 408/13/03/05/2009). All patients had given informed consent for sample collection and analysis. In the case of a minor, a parent gave the consent. All research conformed with the principles of the Declaration of Helsinki. The tumor percentages (>95%) were verified with hematoxylin and eosin stainings. All patients were mutation negative for the established germline mutations associated with pituitary neoplasia (see the Supplementary Methods). Seven tumors were Gsp+ and 14 tumors Gsp−. Seven patients have hormonally active disease and are treated with postoperative somatostatin analogue therapy (ST3, ST6, ST13, ST16, ST17, ST19, ST21), one (ST6) of them is on somatostatin–cabergoline combination therapy and two (ST3, ST17) on somatostatin–pegvisomant combination therapy. Currently, 14 patients are in hormonal remission, whereas three noncompliant patients (ST3, ST17, ST22) are not, and current medical therapy of four patients (ST2, ST8, ST14, ST15) is not known (Table 1).

WGS, SCNA, and gene expression profiling

Genomic DNA was extracted by FastDNA Spin Kit (MP Biomedicals; tumors) and DNeasy Blood and Tissue Kit (Qiagen; blood). The aryl hydrocarbon receptor interacting protein (AIP) and Gsp mutation status was identified by capillary sequencing as described earlier (6). The WGS genomic DNA libraries were prepared according to Illumina PE sequencing protocols and sequenced to at least 40× median coverage on the Illumina HiSeq 2000 platform (2×100 bp PE; Beijing Genomics Institute, BGI Tech Solutions Co., Ltd.). A genome-wide analysis of tumors for somatic SNVs and SVs was performed as described previously (8). The somatic variants in tumors ST2-12 were identified by filtering against a patient-matched blood sample. For the Gsp+ tumors ST13, ST16, ST18-ST20 and ST22, patient-matched germline variants were not available, and somatic variants were identified.
by filtering against all variants in gnomAD (v2.0.1; https://gnomad.broadinstitute.org), 1000 Genomes Project (phase III, 2013/2012; http://www.internationalgenome.org/home). Sequencing Initiative Suomi (SISu, accessed on March 2016; http://www.sisuproject.fi/), and an in-house collection of 339 normal tissue WGS samples. The remaining variants were filtered to a minimum coverage of 10 reads, minimum alternative coverage of six, and minimum quality score of 40 (phred-scale).

SCNA analysis was performed using SNP arrays (1 kGP HumanOmni2.5-8 BeadChip, Illumina, Inc.). Analysis was performed as described previously (8) comparing individuals’ tumor sample with its corresponding normal blood derived DNA using Genomics Suite v.6.5 (Partek) with a GC-wave correction. Genomic instability percentage (GIP%) was determined by dividing the number of altered chromosomal arms in the tumor by the total number of chromosomal arms.

RNA was extracted with RNaseasy Mini Kit (Qiagen). Expression profiles were generated using GeneChip Human Transcriptome Array 2.0 array (Thermo Fisher Scientific). cDNA synthesis, labeling, and hybridization was performed according to the manufacturer’s instructions. Quality control, normalization, and analysis of data were carried out using Transcriptome Analysis Console v 3.0 (Thermo Fisher Scientific). Unsupervised hierarchical clustering of 1,000 probes with the largest variance was done using cosine distance with bottom-up average linking. The annotation file HTA_2.0. na35.2.hg19.transcript and ANOVA were used to determine differentially expressed genes between tumor groups. Differentially expressed genes were filtered using FDR <0.5% and fold change |FC| > 2.

Bisulfite sequencing and data processing
The target region bisulfite sequencing (TBS) of somatotropinomas was performed utilizing the SureSelectXT Human Methyl. Seq (Agilent Technologies, Inc.) target enrichment system. Illumina paired-end sequencing for libraries was done using 126 base-pair read length and the HiSeq2500 platform (Illumina, Inc.; Beijing Genomics Institute, BGI Tech Solutions Co., Ltd.). The raw TBS data were preprocessed with the Bismark (v0.16.3) pipeline, Bowtie2 (v2.3.0), and the human reference genome (UCSC hg19). A total of 8,493,667 CpGs were observed with a minimum coverage of two, out of which 4,527,285 CpGs passed the minimum coverage in at least four tumors. CpG methylation levels were quantified using bsseq (v1.10.0) and DSS (v2.14.0) as follows. An unsupervised, genome-wide analysis was done using bsseq quantification of the CpG methylation levels: the TBS target regions (N = 350,539) were filtered to a minimum of three CpGs that passed minimum coverage (N = 198,649). The default bsseq smoothing was applied to quantify the methylation level of each target region. An unsupervised hierarchical clustering of 50,000 TBS regions with the largest variance was done using cosine distance with bottom-up average linking. Supervised analysis of differentially methylated regions (DMR) used the default DSS smoothing to test for a minimum mean methylation difference of 0.2 in a two-group comparison. The DMRs were filtered with the default DSS settings (a minimum of 3 CpGs, minimum 50 bps and P < 10^-5).

The gene annotation and their genomic coordinates were based on Ensemble (v82, hg19). The promoter regions were defined as going from –1 kb to +2 kb relative to the transcription starting site (TSS), gene bodies (+2 kb, relative to the TSS, to the end of the gene). Other genomic annotations were downloaded from the UCSC table browser (accessed on August 2017) for ENCODE enhancer regions (6 human cell lines; awg segmentation combined), DNasel Hypersensitivity Clusters (ENCODE v3), ENCODE transcription factor clusters (TFBS clusters v3; 161 factors) including the CCCTC-binding factor (CTCF) sites, and CpG islands (16). CpG island shores were composed of 2 kb upstream and downstream regions flanking the CpG islands. Methylation differences with regards to replication timing were annotated based on HeLa cell line data (17).

CpG methylation levels were quantified within [0, 1], where 0 and 1 correspond to total absence and presence of the mark, respectively.

Expression quantitative methylation analysis
Expression quantitative methylation (eQTM) analysis was used to identify association between methylation and gene expression levels. The DMRs and their nearby genes were tested for association, also known as cis-eQTM (18), using MatrixEQTL (v2.1.1). The cis-eQTM were filtered to a maximum 20 Kbp distance between the gene and DMR. The resulting associations were filtered to FDR <5%.

Pathway analyses
The pathway data were generated with Ingenuity Pathways Analyses (IPA) software (https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis). IPA Summer 2018 Release). The data were mapped into relevant pathways based on their functional annotation and known molecular interactions in Ingenuity’s Knowledge Base (IPKB). The –log of P value were calculated by Fisher exact test. A dataset derived from expression arrays along with the corresponding FC and FDR P values was uploaded into IPA. The eQTM dataset was mapped into relevant pathways in a similar manner.

IHC
Formalin-fixed paraffin-embedded sections (5 μm) of pituitary tumors were studied. Ki-67 (MIB-1) and PTTG1 (pituitary tumor transformation gene 1) IHCs were performed as described earlier (8). Other antibodies used were protein tyrosine phosphatase, receptor type D (PTPRD) rabbit Anti-PTPRD (HPA054822, 1:300; Sigma-Aldrich), protein tyrosine phosphatase, receptor type K (PTPRK) rabbit Anti-PTPRK (HPA054822, 1:400; Sigma-Aldrich), retinoblastoma (RB1) rabbit Anti-RB1 (HPA050082, 1:500; Sigma-Aldrich), and Anti-Rb (phosphor-S780; ab47763, 1:70; Abcam). Antimouse/rabbit/rat secondary antibody, Poly-HRP-GAM/R/R (DVPB55HRP, Immunologic) and DAB chromogen (Thermo Fisher Scientific) were used for detection. Ki-67 and PTTG1 scores were obtained by calculating the average percentage of stained cells among the tumor cell population. Scores were evaluated using a cell counting tool with a grid of EVOS XL Color imaging systems microscope with at 40x lens (Thermo Fisher Scientific) from several sections (3–8 sections/tumor and ~1,400–3,700 cells/tumor). PTPRD, PTPRK, and RB1 proteins were evaluated semiquantitatively by estimating both fractions of immunopositive cells as well as scoring the staining intensities (0, negative; 1, weak; 2, moderate; 3, strong). For scoring details, see the Supplementary Methods.
Results
Somatic SNV and indel background of Gsp+ somatotropinomas
The somatic landscape of SNVs and SVs of the tumors ST2-ST12 was reported previously (8). The tumors had an average of 2.3 coding region SNVs per tumor, with GNAS being the only recurrently mutated gene. Here, we examined the somatic SNVs and SVs of six additional Gsp+ tumors: ST13, ST16, ST18-20, and ST22. Supplementary Table S1 gives all the coding region (missense, premature stop codon, frameshift) variants that passed the somatic filtering. These additional Gsp+ tumors had an excess of somatic variants, in total 92 coding region SNVs and on average 15.3 SNVs per tumor, simply because rare germline variants may have passed the population-based filtering. The majority of the somatic variants (47 SNVs) arise from ST22 due to patient’s Italian ancestry being underrepresented among the population-based controls. Among the 21 tumors studied the only recurrently mutated gene was GNAS. No focal deletions or complex chromosomal rearrangements were observed with exception of ST3, a previously reported Gsp+ tumor (Supplementary Fig. S1; ref. 8).

SCNA and expression profiling differences in CA- and CS-tumors
ST5 data from 21 somatotropinoma normal/tumor pairs was used for SCNA analysis. Results of eleven normal/tumor pairs (ST2-ST12) were from our earlier work (8). Analyses revealed that 12 Gsp+ tumors contained frequent and recurrent (≥4 tumors share the event) chromosomal deletions (chr 1, 4, 6, 14, 15, 16, 18, 22). Also gains (chr 5, 7, 9, 19, 20) of entire chromosomes were detected, although with considerably lower frequency (Supplementary Fig. S1). Copy-neutral LOH or homozygous deletions were not observed. All detected chromosomal gains were duplications of a single chromosome. Gsp+ tumors contained limited amount of aneuploidy, mostly gains of single chromosomes. An exception was a tumor ST4 with genetic instability (GI%) 22% (Table 1; Supplementary Fig. S1). In addition, two Gsp+ tumors (ST1 and ST6) were totally aneuploidy-free. GI%-did not correlate with clinical variables (Supplementary Table S2). To test whether the recurrent aneuploidy is statistically significant, the permutation test was employed (8). The test indicated that the observed recurrent aneuploidy indicates selective advantage during tumorigenesis rather than random copy number alteration (permutation test P < 10^-4; Supplementary Fig. S2).

An unsupervised hierarchical clustering analysis of gene expression demonstrated that aneuploidy was the major determinant of somatotropinoma subtypes. Tumors clustered according to the aneuploidy rate as follows (Fig. 1A; principal components analysis in Supplementary Fig. S3). Twelve Gsp+ tumors with aneuploidy, from now on called CA (chromosome aneuploidy)-tumors, clustered together. Accordingly, chromosome stable (CS)-tumors with limited amount or no aneuploidy (7 Gsp+ and 2 Gsp- ) formed their own distinct group. Because CA- and CS-tumors had their own expression signatures (Fig. 1A) expression profile comparison was performed between CA- and CS-tumors. We identified 881 differentially expressed transcripts (FDR < 5%, |FC| > 2; Supplementary Table S3). Integration of expression and SCNA data revealed that 69.8% of differentially expressed transcripts locate at chromosomes with recurrent aneuploidy (≥4 tumors with shared aneuploidy) from which 81.6% positively correlate with the chromosomal copy number change.

To understand the biological relevance of differentially expressed genes in CA-somatotropinomas, pathway analysis was performed. The top canonical pathway emerged from the tumor subtype-specific expression was the PKA pathway (Fig. 1B; Supplementary Table S4). In this cAMP-mediated signaling pathway a majority of molecules were upregulated in CS-tumor group indicating activated PKA signaling (Fig. 2; Table 2). The major inhibitors of cAMP levels (19), phosphodiesterases (PDE) were upregulated in CS-tumor group and seven protein tyrosine phosphatase (PTP) receptors were differentially expressed between tumor groups (Table 2).

The expression pathway analysis also highlighted several pathways associated with cell-cycle regulation and explicitly in the retinoblastoma 1/E2F transcription factor (RB1/E2F)-mediated G1-S-checkpoint transition. Among the enriched pathways were molecular mechanisms of cancer, chronic myeloid leukemia, glioma, glioblastoma multiforme, and cell cycle: G1-S checkpoint regulation (Fig. 1B; Supplementary Table S4). The genes showing expression differences between tumor groups accumulated in the RB1/E2F-mediated cell-cycle regulation and the subsequent G1-S-checkpoint transition indicating difference in mitotic progression (Fig. 1C). E2F4 transcription factor, a regulator of cell cycle (20), was downregulated in CA-tumor group. By contrast, histone deacetylase 5 (HDAC5) and RB transcriptional co-repressor like 1 (RBL1/p107), both repressors of E2F family members, were upregulated in CA-tumor group when compared to CS-tumors. Also, the anti-immtogenic growth factor TGFβ2/SMAD3 signaling was downregulated in CA-tumor group (Fig. 1C; Table 3).

IHC
Because RB1 is the major component of the complex regulating G1-S phase transition, protein levels of total- and phosphorylated-RB1 were semiquantitatively assessed in tumors using IHC. In all tumors >90% of nuclei showed positive total- and phosphorylated-RB1 staining. Because fractions of stained cells were comparable between tumor groups, staining intensities were compared. In both CA- and CS-tumor groups was seen weak to moderate nuclear immunoreactivity of total-RB1 (CA- vs. CS-tumors: 1.67 vs. 1.78, P = 0.6, Student t-test). In addition, some occasional cytoplasmic total-RB1 staining was detected in both tumor groups. Phospho-RB1 showed weak to moderate nuclear staining in both tumor groups (CA- and CS- tumors: 1.59 vs. 1.67, P = 0.71; Supplementary Table S3; Supplementary Fig. S4).

To test pathway analysis findings of the PKA signaling (Fig. 2) at the protein level, PTPRD and PTPRK IHCs were performed. For this, we used tumor material from four CA- (ST10, ST12, ST18, ST19) and three CS-tumors (ST15, ST17, ST21). PTPRD showed negative immunostaining in CA-tumors (Gsp+ ) and moderate immunoreactivity in CS-adenomas (Gsp- ); Supplementary Fig. 5A). PTPRK immunostaining was negative or weak in CA-tumors and moderate in CS-tumors (Supplementary Fig. S5B). In immunopositive tumors, both PTPRD and PTPRK localized mainly in cytoplasm and >90% of cells gave positive immunostaining.

To investigate the role of PTTG1 in the formation of pituitary neoplasia and proliferation rates in these tumors, PTTG1 and Ki-67 immunostainings were performed. Ki-67 and PTTG1 proteins...
Figure 1.

A, Gene expression of somatotropinomas clustered according to the aneuploidy rate. Left, result of unsupervised hierarchical clustering of expression array data from 21 somatotropinomas; middle, patients’ age at diagnosis, gender, and Gsp mutation status; right, somatic copy number aberrations. Supplementary: Supplementary Fig. S1 shows all somatic chromosomal aberrations in more detail. Of note, the chr 1p of ST3 contains chromothripsis event (8).

B, Enriched pathways result from differentially expressed genes (CA- vs. CS-tumors). Supplementary Table S4 shows the complete list of significantly (P < 0.05) enriched expression pathways and genes. The blue horizontal bars denote the association P values for each pathway on a logarithmic scale (dashed vertical line at P = 0.05). The ratio between the number of query genes found and total number of genes in a pathway is shown in orange. C, The enriched G1–S signaling pathway. The colored molecules identified as differentially regulated in CA- versus CS-tumor groups. FCs and P values are listed in Table 3. Orange label, upregulated; green label, downregulated.
were expressed in all tumors. Ki-67 gave a clear nuclear immunostaining. There was no significant difference in Ki-67 scores between tumor groups ($x = 2.7 \pm 1.6$ vs. $1.7 \pm 1.2$; $P = 0.27$; Table 1). PTTG1 immunopositive cells showed both nuclear and cytoplasmic localization of the protein. Most of the cells showed predominant cytoplasmic staining, although there were also cells with predominant nuclear localization of PTTG1 in both tumor groups (Supplementary Fig. S6). All stained cells were scored and number of immunopositive cells were significantly more abundant in CA-tumors compared with CS-adenomas ($x = 1.8 \pm 1$ vs. $0.9 \pm 0.5$; $P = 0.016$; Wilcoxon–Mann–Whitney; Table 1). PTTG1 RNA expression levels, emerging mainly from nonproliferating cells, were comparable between CA- and CS-tumor groups ($FC = 1.05$, $P = 0.733$). As has been seen earlier ($8, 21$), the PTTG1 protein levels correlated with Ki-67 scores ($r = 0.62$, $P = 0.002$; Pearson coefficient).
Table 2. Differentially expressed genes in the PKA signaling pathway (CA- vs. CS-tumors)

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<th>FDR</th>
<th>Type</th>
<th>Symbol</th>
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Abbreviation: FC, expression fold change.

DNA methylation

We surveyed DNA methylation and DMRs in 21 somatotropinomas by targeted bisulfite sequencing (Supplementary Table S6). An unsupervised clustering of 50,000 CpG regions with the largest variance between tumors showed, that apart from ST7 and ST21, the tumors clustered according to the Gsp mutation status (Fig. 3A; principal components analysis in Supplementary Fig. S7). The genome-wide distributions of CpG methylation levels for different genomic contexts revealed that methylation levels of promoter regions were comparable across tumors (Fig. 3A). The rest of the annotated regions showed hypomethylation of Gsp+ tumors (see below for the supervised analysis of Gsp+ and Gsp−). Eighteen percent of the differentially expressed (CA- vs. CS) transcripts (119/670 coding transcript clusters; Supplementary Table S3) were correlated with DNA methylation.

DNA methylation patterns are maintained and regulated by DNA methyltransferases (DNMT), including DNMT1, DNMT3A, and DNMT3B (22). We examined tumor-specific associations of DNMT expressions and median CpG methylation and found that CpG methylation rates correlated with the DNMT1 expression (Spearman rank correlation 0.49; P = 0.025; Supplementary Fig. S8), while DNMT3A (0.02, P = 0.929) and DNMT3B (0.24, P = 0.2294) did not show correlation.

DNA methylation associates also with replication timing (17). Supplementary Figure S9 shows an overview of CpG methylation at different quartiles of replication timing. Majority of the tumors displayed the expected hypomethylation of late replicating regions (Spearman ρ < 0 and P < 0.019; Supplementary Table S7). The Gsp− tumors ST10, ST11, ST16, and ST20 had an outstanding, positive correlation to replication time (Spearman ρ >0 and P < 0.018), which suggests methylation maintenance also at late replicating regions. No clinical associations were found to explain the methylation maintenance difference in these four tumors (Table 1).

Because the Gsp mutation status was the major factor behind the DNA methylation rates and profiles across tumors, DMRs were determined between Gsp+ versus Gsp− somatotropinomas. Altogether, we found 1,369 DMRs out of which 1,339 (97.8%) were hypomethylated in Gsp+ tumors: see Supplementary Table S8 for a complete list of all 1,369 regions' genomic coordinates and annotation of nearby (-20 Kbp upstream; 2 Kbp downstream) genes (1,560 gene annotations). Supplementary Table S8 is sorted by the absolute value of the test statistic, where negative direction denotes hypomethylation among the Gsp+ tumors compared to the Gsp− tumors. Both the outstanding number of DMRs and the enrichment of Gsp+ tumors toward hypomethylation can likely be attributed to the genome-wide CpG methylation characteristics between the tumor types (see the unsupervised analysis and Fig. 3A). The DMRs did not enrich among the aneuploidy chromosomes, 51% of the DMRs reside at recurrent aneuploidy, while the expected proportion was 55% based on the distribution of the TBS regions.

In addition to the DMR analysis, we also examined the genome-wide methylation profiles between Gsp+ and Gsp− tumors in different genomic contexts. Supplementary Table 3. G−S checkpoint regulation enriched genes from expression pathway analysis (CA- vs. CS-tumors)

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Entrez gene name</th>
<th>FC</th>
<th>FDR</th>
<th>Type</th>
<th>Symbol</th>
<th>Entrez gene name</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2F4</td>
<td>E2F transcription factor 4</td>
<td>−2.230</td>
<td>0.030</td>
<td>Transcription regulator</td>
<td>E2F</td>
<td>E2F transcription factor 4</td>
</tr>
<tr>
<td>HDAC5</td>
<td>Histone deacetylase 5</td>
<td>2.180</td>
<td>0.004</td>
<td>Transcription regulator</td>
<td>HDAC</td>
<td>Histone deacetylase 5</td>
</tr>
<tr>
<td>RBL1</td>
<td>RB transcriptional coactivator like 1</td>
<td>2.080</td>
<td>0.006</td>
<td>Transcription regulator</td>
<td>RBL1</td>
<td>RB transcriptional coactivator like 1</td>
</tr>
<tr>
<td>RBK2B8</td>
<td>RB binding protein 8, endonuclease</td>
<td>−4.06</td>
<td>0.004</td>
<td>Enzyme</td>
<td>RBK2B8</td>
<td>RB binding protein 8, endonuclease</td>
</tr>
<tr>
<td>SMAD3</td>
<td>SMAD family member 3</td>
<td>−3.020</td>
<td>0.010</td>
<td>Transcription regulator</td>
<td>SMAD3</td>
<td>SMAD family member 3</td>
</tr>
<tr>
<td>TGFβ2</td>
<td>Transforming growth factor β2</td>
<td>−2.120</td>
<td>0.045</td>
<td>Growth factor</td>
<td>TGFβ2</td>
<td>Transforming growth factor β2</td>
</tr>
</tbody>
</table>

Abbreviation: FC, expression fold change.
Figure 3.

A, CpG methylation of somatotropinomas clustered according to the Gsp mutation status. Unsupervised hierarchical clustering of CpG methylation data from 21 somatotropinoma samples (based on 50,000 regions with the largest variance between tumors); middle, patient’s age at diagnosis, gender, and Gsp mutation status; right, distribution of estimated CpG methylation levels for each genomic context: gene body (from –2 kb relative to the TSS to the end of gene), promoter (–150 to +2 kb relative to the TSS), CpG island/shore, enhancer, DNase (DNaseI Hypersensitivity Cluster), and TFBS (Transcription factor cluster; see Materials and Methods for details). Methylation levels are quantified by value ranging from zero (unmethylated) to one (fully methylated).

B, Pathway analyses from cis-eQTM. Supplementary Table S10 shows the list of significantly (<0.05) enriched cis-eQTM pathways and genes. The blue horizontal bars denote the association P values for each pathway on a logarithmic scale (dashed vertical line at P = 0.05). The ratio between the number of query genes found and total number of genes in a pathway is shown in orange.

C, The enriched CREB signaling in neurons pathway. The colored molecules identified as differentially expressed Gsp+ versus Gsp− tumors. eQTM and FDR values are listed in Table 4. Orange label, upregulated; green label, downregulated.
Table 4. Differentially regulated genes in the CREB signaling pathway (Gsp+ vs. Gsp–)

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Entrez gene</th>
<th>eQTMj</th>
<th>FDR</th>
<th>Type</th>
<th>Symbol in pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>CACNA1A</td>
<td>Calcium voltage-gated channel subunit alpha A</td>
<td>6.10</td>
<td>0.001</td>
<td>Ion channel</td>
<td>CaCn</td>
</tr>
<tr>
<td>CACNA1E</td>
<td>Calcium voltage-gated channel subunit alpha E</td>
<td>1.73</td>
<td>0.019</td>
<td>Ion channel</td>
<td>CaCn</td>
</tr>
<tr>
<td>CACNG2</td>
<td>Calcium voltage-gated channel auxiliary subunit gamma 2</td>
<td>5.25</td>
<td>0.001</td>
<td>Ion channel</td>
<td>CaCn</td>
</tr>
<tr>
<td>GNAI2</td>
<td>G protein subunit alpha i2</td>
<td>–1.17</td>
<td>0.016</td>
<td>Ion channel</td>
<td>Gp/Gxi</td>
</tr>
<tr>
<td>GNB1</td>
<td>G protein subunit beta 1</td>
<td>–1.40</td>
<td>0.020</td>
<td>Ion channel</td>
<td>Gb</td>
</tr>
</tbody>
</table>

Abbreviation: eQTM, expression quantitative trait methylation, a correlation between gene expression and methylation.

Discussion

Pituitary tumors are slowly growing benign neoplasia with a low mitotic activity due to senescence. Somatic SNVs and SVs are rarely found in these tumors, indicating that also other mechanisms are driving tumorigenesis (7, 8, 9, 10, 11, 12). Aneuploidy is a common feature in solid tumors, and it provides cancer cells a mechanism to lose tumor suppressors and gain extra copies of oncogenes (13, 14). However, a causal relationship between aneuploidy and tumorigenesis as well as genes/pathways that are deregulated by aneuploidy are still incompletely characterized (27). Aneuploidy is a relatively common event in somatotropinomas (8, 12, 28). In the current study, we were able to confirm that somatotropinomas create two subtypes associated with distinct aneuploidy rates and unique transcription profiles. The CA-tumor subtype contained Gsp+ tumors characterized by frequent and recurrent aneuploidy. Recurrent aneuploidy has been associated earlier with more malignant tumors (29), suggesting a selective advantage and role in the tumor evolution in these cancer types. The other subtype, CS-tumors, contained all Gsp+ tumors together with two Gsp– adenomas. These tumors were either totally aneuploidy-free or displayed only single chromosome number changes, indicating that expression changes caused by chromosome copy number alterations are poorly tolerated in this tumor subtype.

In some tumor types, aneuploidy is associated with tumor recurrence, and drug resistance (13, 14). In the current study, clinical features of the patients did not associate with aneuploidy (Supplementary Table S2). Moreover, larger studies have shown that there is no difference in clinical characteristics and outcome of the patients with or without Gsp mutation (6, 19), indicating that most aneuploid Gsp+ tumors do not progress toward aggressive disease.

Because GH-secreting cells constitute only up to 45% of normal anterior pituitary cells (30) and because tumor groups had their own expression signatures (Fig. 1A), expression profile comparison was performed between CA- and CS-tumor groups. In addition, no normal anterior pituitary lobe tissue was available for the study. Differentially expressed genes in CA- and CS-tumors enriched most significantly in the PKA signaling. It is well established that oncogenic Gsp mutations activate the cAMP-dependent PKA pathway (5). Therefore, the result reflects the Gsp+ tumor-induced activation of PKA signaling in the CS-tumor group. In addition to the previously identified Gsp+ tumor-associated molecules (19), we found that many protein tyrosine phosphatase (PTP) receptors were differentially expressed in CA- and CS-tumors (Supplementary Table S8). In a supervised association analysis of eQTMs, 392 DMR regions (98%) were tolerated in this tumor subtype.

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RBI/E2F complex has a major role in the cell-cycle regulation. It controls the G1–S phase transition during the cell cycle and is regulated by the RBI pocket proteins (RBI, RBL1/p107, RBL2/p130) and E2F transcription factors. Dysregulated G1–S-phase transition promotes tumor formation and may give rise to aneuploidy. Inactivation of RBI through phosphorylation leads release of E2F transcription factors and subsequent cell-cycle progression. It has been demonstrated that dysregulated RBI/E2F complex is involved in the pituitary tumorigenesis. Mice with heterozygous Rbi mutation develop pituitary adenomas with almost full penetrance, while loss of E2F1 reduces the frequency of pituitary tumors in Rbi+/− mice (20, 32, 33). Our expression data showed that RBI/E2F–mediated G1–S checkpoint signaling is differentially regulated between the tumor groups. The CA-tumor group displayed downregulation of E2F4, whereas E2F-repressors HDAC5 and RBL1/p107 (34) were upregulated.
Interestingly, apart from HDAC5 (17q21), all differentially expressed G1–S–related genes, E2F4 (16q22), RB1L (20q11), and TCF7L2 (1q11), SMAD3 (15q22) locate on chromosomes with recurrent aneuploidy and their expression correlated with the direction of aneuploidy (Supplementary Fig S1). Although E2F4 was differently expressed in CA- and CS-tumors, we did not observe differences in fractions of immunopositive cells or protein levels of total- and phosphorylated-RB1 between CA- and CS-tumor groups. E2F4 has traditionally been categorized as a transcriptional repressor, but more recently it was demonstrated that in some tissue types E2F4 can also act as an activator of proliferation (35). The transcriptional role of E2F4 in somatotropinomas is not known. Therefore, the observed differently expressed RB1/E2F4 complex between CA- and CS-somatotropinoma subtypes can reflect dysregulation of cell cycle progression in either one of the tumor groups.

The pituitary tumor-transforming 1 gene (PTTG1; 5q33) is a mitotic checkpoint protein that regulates a sister chromatid segregation during mitosis as well as genes encoding G1–S and G2–M phase proteins (36–38). RNA and protein levels of PTTG1 exhibit a cell cycle–dependent expression pattern, being highest at G2–M phase and attenuated after mitosis. PTTG1 is expressed in all types of pituitary tumors (39, 40). It has been shown that both loss and overexpression of PTTG1 lead to cell cycle dysregulation and promote aneuploidy and G1–S cell-cycle arrest induced senescence. A mouse model with overexpressed Pttg1 develop pituitary hyperplasia and adenomas, whereas knockout Pttg1–/– mice exhibit pituitary hypoplasia (41, 42). Crossbreeding of overexpressed Pttg1 animal with heterozygous Rb1+/– mice increases penetrance of pituitary tumors. In contrast, crossbreeding of Pttg1 knockout animal with Rb1+/– mice showed decreased pituitary tumor formation, indicating that the absence of Pttg1 has a protective effect on pituitary tumor formation (41–45).

In the current study, we showed that CA-tumors with recurrent aneuploidy exhibit more abundant PTTG1 protein levels. This finding together with the known biological function of PTTG1 in pituitary tumorigenesis (36, 39, 41, 43, 44) imply that elevated PTTG1 protein levels exhibit more abundant PTTG1 protein levels. This tendency to cluster according to the Gsp genotype suggests involvement of somatotropinomas. Interestingly, apart from HDAC5 (17q21), all differentially expressed G1–S–related genes, E2F4 (16q22), RB1L (20q11), and TCF7L2 (1q11), SMAD3 (15q22) locate on chromosomes with recurrent aneuploidy and their expression correlated with the direction of aneuploidy (Supplementary Fig S1). Although E2F4 was differently expressed in CA- and CS-tumors, we did not observe differences in fractions of immunopositive cells or protein levels of total- and phosphorylated-RB1 between CA- and CS-tumor groups. E2F4 has traditionally been categorized as a transcriptional repressor, but more recently it was demonstrated that in some tissue types E2F4 can also act as an activator of proliferation (35). The transcriptional role of E2F4 in somatotropinomas is not known. Therefore, the observed differently expressed RB1/E2F4 complex between CA- and CS-somatotropinoma subtypes can reflect dysregulation of cell cycle progression in either one of the tumor groups.

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In the current study, we showed that CA-tumors with recurrent aneuploidy exhibit more abundant PTTG1 protein levels. This finding together with the known biological function of PTTG1 in pituitary tumorigenesis (36, 39, 41, 43, 44) imply that elevated PTTG1 levels may be involved in the development of aneuploidy in somatotropinomas. During the initial steps of tumorigenesis, slowly accumulating aneuploidy can mediate excessive proliferation by changing gene expressions and modulating functions of pituitary tumor driver pathways. Eventually, however, recurrent aneuploidy leads to mitotic stress and senescence via altered levels of proteins involved in the RB1/E2F–mediated G1–S cell-cycle progression. Thus, in CA-somatotropinomas aneuploidy may underlie both the tumor formation as well as escape from aggressive growth and malignancy.

Interestingly, PTTG1 seems to be a downstream target of E2F transcription factor family (33). However, the regulatory mechanisms of PTTG1 are only partially elucidated and further studies are required to evaluate the exact regulatory mechanisms of PTTG1 in pituitary tumors.

Alterations of DNA methylation have been recognized as an important component of tumor development and progression of cancer through different mechanisms. It has been shown that pituitary tumors have their own distinct DNA methylation profile without overlapping with other sellar region tumors (12). The current work shows that DNA methylation of somatotropinomas tends to cluster according to the Gsp mutation status. DNA methylation levels can have longitudinal changes due to epigenetic reprogramming during tumorigenesis (46), which may explain the observed misclustered tumors in our sample set. In general, Gsp+ tumors were hypomethylated when compared with Gsp– tumors and distributions of methylation levels for different genomic contexts across tumors revealed distinct Gsp genotype-specific methylation profiles. DNM1T1 is a methyltransferase enzyme, which maintains DNA methylation during cell replication and aberrant expression of DNM1T1 is involved in tumor transformation and progression in many cancer types (22, 47). In the current study, expression of DNM1T1 was found to be positively correlated with tumor-specific methylation levels, suggesting involvement of DNM1T1 in the tumorigenesis of somatotropinomas.

Gsp genotype-specific DNA methylation profiles indicate that different molecular mechanisms are involved in the development and progression of Gsp+ and Gsp– pituitary tumors. The integration of DNA methylation and gene expressions indicated that the inhibitory Gαi protein (Gαi) signaling, together with the voltage-gated calcium channel (CaCn) transducer signaling seem to be the major biological functions differentially regulated via DNA methylation in these tumor subtypes. In Gsp+ tumors Gαi signaling was activated through overexpression of Gαi2 (GNAI2) and Gβ1 (GNB1), whereas CaCn subunits were downregulated. Both of these signaling cascades are involved in the regulation of cAMP response. Inhibitory Gαi proteins most notably inhibit receptor-dependent cAMP synthesis (48) and CaCn signaling stimulates the cAMP response element-binding (CREB) protein, a main downstream target of mitogenic effect of cAMP (26, 49, 50).

Thus, activated Gαi and downregulated CaCn may reflect the adaptive response to elevated cAMP levels caused by GNAS (Gαs) mutation and prevent the excessive cellular proliferation in Gsp+ tumors. We have earlier shown that dysfunctional Gαi signaling and particularly the reduced Gαi2 protein levels contribute to the development of aryl hydrocarbon receptor interacting protein (AIP) germline mutation associated somatotropinomas (25). This study, however, implies for the first time that Gαi signaling is upregulated in Gsp+ somatotropinomas with constitutively activated cAMP synthesis.

The systematic characterization of the somatic landscape using genomic, epigenomic, and transcriptomic data across Gsp+ and Gsp– somatotropinomas highlighted tumor subtypes and subtype-specific mechanisms of tumorigenesis. The study suggest that aneuploidy through modulated driver pathways may be a causative mechanism for tumorigenesis in Gsp– adenomas, whereas Gsp+ tumors are characterized by DNA methylation controlled Gαi–CaCn signaling, possibly in response to the mitogenic cAMP signaling caused by GNAS mutation. Although subsequent studies are needed to fully characterize the molecular mechanisms resulting from aneuploidy-induced gene expression changes and the exact role of PTTG1 in the formation of recurrent aneuploidy in pituitary neoplasia, the work presented here provides valuable new information about subtype-specific pituitary tumorigenesis. Moreover, these findings may help to elucidate the mechanisms of aneuploidy also in other tumor types.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

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Development of methodology: N. Valimäki, A. Karhu

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Välimäki, C. Schalin-Jantti, A. Paetau, A. Karhu

Writing, review, and/or revision of the manuscript: N. Välimäki, C. Schalin-Jantti, A. Karppinen, A. Paetau, L. Kivipelto, L.A. Aaltonen, A. Karhu

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Välimäki, A. Karhu

Study supervision: L.A. Aaltonen, A. Karhu

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References


Secreting Pituitary Tumors
Genetic and Epigenetic Characterization of Growth Hormone–Secreting Pituitary Tumors

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