Gene and protein expression in pituitary corticotroph adenomas: a systematic review of the literature

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OBJECT Functional corticotroph pituitary adenomas (PAs) secrete adrenocorticotropic hormone (ACTH) and are the cause of Cushing's disease, which accounts for 70% of all cases of Cushing's syndrome. Current classification systems for PAs rely primarily on laboratory hormone findings, tumor size and morphology, invasiveness, and immunohistochemical findings. Likewise, drug development for functional ACTH-secreting PAs (ACTH-PAs) is limited and has focused largely on blocking the production or downstream effects of excess cortisol. The authors aimed to summarize the findings from previous studies that explored gene and protein expression of ACTH-PAs to prioritize potential genetic and protein targets for improved molecular diagnosis and treatment of Cushing's disease.

METHODS A systematic literature review was performed using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. A PubMed search of select medical subject heading (MeSH) terms was performed to identify all studies that reported gene- and protein-expression findings in ACTH-PAs from January 1, 1990, to August 24, 2014, the day the search was performed. The inclusion criteria were studies on functional ACTH-PAs compared with normal pituitary glands, on human PA tissue only, with any method of analysis, and published in the English language. Studies using anything other than resected PA tissue, those that compared other adenoma types, those with- pared with normal pituitary glands, on human PA tissue only, with any method of analysis, and published in the English language. Studies using anything other than resected PA tissue, those that compared other adenoma types, those without baseline expression data, or those in which any pretreatment was delivered before analysis were excluded.

RESULTS The primary search returned 1371 abstracts, of which 307 were found to be relevant. Of those, 178 were selected for secondary full-text analysis. Of these, 64 articles met the inclusion criteria and an additional 4 studies were identified from outside the search for a total of 68 included studies. Compared with the normal pituitary gland, significant gene overexpression in 43 genes and 22 proteins was reported, and gene underexpression in 58 genes and 15 proteins was reported. Immunohistochemistry was used in 39 of the studies, and reverse transcriptase polymerase chain reaction was used in 26 of the studies, primarily, and as validation for 4 others. Thirteen studies used both immunohistochemistry and reverse transcriptase polymerase chain reaction. Other methods used included microarray, in situ hybridization, Northern blot analysis, and Western blot analysis. Expression of prioritized genes emphasized in multiple studies were often validated on both the gene and protein levels. Genes/proteins found to be overexpressed in ACTH-PAs relative to the normal pituitary gland included hPTTG1/sercin, NEUROD1/NeuroD1 (Beta2), HSD11B2/11β-hydroxysteroid dehydrogenase 2, AKT/Akt, protein kinase B, and CCND1/cyclin D1. Candidate genes/proteins found to be underexpressed in ACTH-PAs relative to the normal pituitary gland included CDKN1B/p27Kip1, CDKN2A/p16, KISS1/kisspeptin, ACTHR/ACTH-R, and miR-493.

CONCLUSIONS On the basis of the authors’ systematic review, many significant gene and protein targets that may contribute to tumorigenesis, invasion, and hormone production/secretion of ACTH have been identified and validated in ACTH-PAs. Many of these potential targets have not been fully analyzed for their therapeutic and diagnostic potential but may represent candidate molecular targets for biomarker development and drug targeting. This review may help catalyze additional research efforts using modern profiling and sequencing techniques and alteration of gene expression.

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KEY WORDS gene expression; protein expression; corticotroph; ACTH; pituitary adenoma
Pituitary adenomas (PAs) are common tumors with an overall prevalence in the general US population estimated at 16.7%. Corticotroph adenomas, comprising functional and silent corticotroph adenomas, represent approximately 10%–15% of all PAs. Functional adrenocorticotropic hormone–secreting PAs (ACTH-PAs) secrete inappropriate amounts of ACTH, which results in disorderly and excessive production of cortisol by the adrenal gland. Functional ACTH-PAs (Cushing’s disease) are the most common cause of Cushing’s syndrome (hypercortisolism from any source) and account for an estimated 70% of all cases. The prevalence of Cushing’s disease is estimated to be 39 per 1,000,000 people (approximately 12,000 people affected in the United States alone). This number, however, may be much higher, given that Cushing’s disease is frequently misdiagnosed and the diagnosis is often delayed.

Current classification systems for PAs are based primarily on secretory characteristics of the tumor but are also classified on the basis of phenotypical characteristics, including tumor size, degree of invasiveness (e.g., Knosp scoring system), and immunohistological findings. The WHO classification system for PAs has been refined to include designations for benign adenoma, atypical adenoma, and pituitary carcinoma on the basis of p53 immunoreactivity, MIB-1 index, mitotic activity, and the absence/presence of metastases. More comprehensive molecular classification systems based on relevant gene expression have not been systematically used to further characterize pituitary tumors.

Transsphenoidal resection remains the first-line treatment for most patients with Cushing’s disease. Radiosurgery, radiation therapy, medical therapy, and bilateral adrenalectomy are second-line treatments often implemented or reserved as adjuvant treatments for patients with refractory Cushing’s disease.

Currently available pharmacological agents for treating functional ACTH-PAs include ketoconazole, mifepristone, and pasireotide. Ketoconazole, which blocks steroid hormone production, is the primary drug used for this purpose despite it not being formally approved for this use. It is nonspecific and can cause significant reduction of androgen levels and hepatic dysfunction. Pasireotide is a novel somatostatin receptor ligand with greater affinity of androgen levels and hepatic dysfunction. Pasireotide is a novel somatostatin receptor ligand with greater affinity.

In the near future, personalized molecular strategies are likely to improve diagnosis and therapeutics in patients with ACTH-PAs. To summarize and consolidate findings pertaining to ACTH-PAs and prioritize targets for further drug development and molecular classification systems for patients with Cushing’s disease, we reviewed the literature to identify gene- and protein-expression findings in ACTH-PAs. In this article, we present the results of a systematic literature review conducted to identify overexpression and underexpression of genes and proteins that are associated with the development, invasion, and hormonal secretion of ACTH-PAs. By collecting, analyzing, and presenting this information, we hope to shed additional light on prioritized genes and molecular mechanisms associated with specific phenotypical outcomes, potential therapeutic targets, and promising areas of further research.

Methods

Overview of Systematic Literature Review

A systematic literature review was performed to identify all published reports that detailed gene-expression changes and proteins in functional ACTH-PAs relative to the normal pituitary gland.

Preliminary Search Strategy and Terms

The primary objective of the search strategy was to identify all contemporary published studies pertaining to gene expression in functional ACTH-PAs. A detailed systematic search strategy was created by informally searching relevant articles and capturing the most common medical subject heading (MeSH) terms. MeSH terms and Boolean functions were used instead of simpler search strings as a means of creating a more focused search along varied criteria. To focus on results from more modern techniques, all search results were limited to those published between January 1, 1990, and August 24, 2014, but no other limitations were initially placed on the data collection. The search of contemporary articles was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. Using the PubMed database, our search involved a string of the following MeSH terms: ((pituitary neoplasms/genetics[MeSH terms]) OR (pituitary neoplasms/pathology[MeSH terms]) OR (pituitary neoplasms/metabolism[MeSH terms]) OR (pituitary neoplasms/chemistry[MeSH terms]) AND (humans[MeSH terms]) AND ((immunohistochemistry[MeSH terms]) OR (reverse transcriptase polymerase chain reaction[MeSH terms]) OR (gene expression regulation, neoplastic[MeSH terms]) OR (gene expression profiling[MeSH terms]) OR (oligonucleotide array sequence analysis[MeSH terms]) OR (microarray analysis[MeSH terms]) OR (gene expression[MeSH terms])).

Inclusion and Exclusion Criteria

Inclusion criteria were the following: 1) functional ACTH-PAs compared with the normal pituitary gland; 2) analyses of human tissue only; 3) any method of measuring and analyzing protein and/or mRNA expression; and 4) studies published in the English language.

Articles were excluded on the basis of the following: 1) use of anything but resected human PA tissue, including primary cultures, cell lines, or transfected cells; 2)
gene-expression comparisons between various PA tumor types or between adenomas and carcinomas; 3) expression or no expression validation without any baseline; or 4) any defined medical pretreatment of the tumor before analysis.

Implementation of the Search Strategy and Study Selection Process

A preliminary review of the search results was conducted. All the articles were initially screened by 1 reviewer (J.S.) for relevancy on the basis of their titles and abstracts. Any report not excluded by this process was included for full-text analysis. The reports were then subjected to full-text analysis using our inclusion and exclusion criteria. The articles were reviewed by 2 readers independently (J.S. and D.P.); in total, 5 articles differed between the 2 analyses and were reconciled by joint review.

Data Collection Process

From the included studies, information of interest primarily concerned gene and/or protein expression and experimental methods and results. The primary outcomes recorded were tumor type(s) analyzed, expression analysis type(s), results of expression analysis, and any relevant commentary (e.g., association with invasiveness).

Potential for Bias

The search and subsequent analyses were intended to be as unbiased as possible. The preliminary search was performed solely on the basis of relevance and time period (after 1990) with no consideration given to the source, author, institution, or other unique identifying criterion. The primary screening process was performed by one author and subsequently reviewed by another author, which potentially introduced a source of bias.

All full-text studies were reviewed independently by 2 readers only on the basis of our inclusion and exclusion criteria. Furthermore, these inclusion and exclusion criteria were defined before beginning the screening process. Discrepancies between the reviewers were resolved using the inclusion and exclusion criteria. Finally, we had no competing interests and received no outside funding from any source for this research.

Quality Assessment

A formal quality assessment was not performed. However, each full-text article we reviewed included an informal review of the materials, methods, and results. Although this was done primarily to satisfy the inclusion and exclusion criteria, it also enabled us to assess the quality of the studies. No studies were excluded on the basis of poor-quality methods or results.

Results

Literature Review

The search yielded 1371 initial results, of which 307 articles were selected for review on the basis of the relevancy of their titles and abstracts. Of the 307 studies that were reviewed primarily, 178 were selected for full-text analysis. After an in-depth review of 178 full reports, 60 were selected for inclusion on the basis of our inclusion and exclusion criteria. Four articles were added to the review from outside of the formal literature review, sourced from the informal search that yielded the MeSH terms detailed earlier. These reports were subjected to and passed the same inclusion and exclusion criteria as those the search generated. After combining both groups, a total of 64 articles were analyzed. A PRISMA diagram detailing the search results is shown in Fig. 1.

In total, 43 genes and 22 proteins were identified as being overexpressed and 58 genes and 15 proteins as underexpressed in functional ACTH-PAs compared with those in normal pituitary gland tissue. Many of the genes were also characterized by immunostaining for the resulting gene product/protein, all of which paralleled the levels of gene expression. The provided protein data refer only to proteins identified via immunohistochemistry (IHC) and/or Western blotting without analysis of the underlying gene expression.

Of the genes, NEURODI1 and hPTTG1 were overexpressed in 3 different studies, whereas HIGD1B and HSD11B2 were overexpressed in 2 studies. Underexpression of CDKN1B, CDKN2A, and let-7 was shown in 4, 2, and 2 studies, respectively. Concerning the proteins, only c-myc was shown by IHC analysis to be overexpressed in more than 1 study. Underexpression of 2 proteins was shown multiple times: p27Kip1 in 4 studies (all by IHC) and p16 in 2 studies (1 by Western blot analysis and 1 by IHC).

The most common method of analysis was IHC, which was used as the primary method in 39 studies. The second most common was reverse transcriptase polymerase chain reaction (RT-PCR), which was used as the primary method in 26 studies and as a validation method in 4 others in which microarray was used as the primary method. It should be noted that 13 studies used RT-PCR and IHC together. Microarray analysis was used in 6 studies, in situ hybridization in 5, Northern blot analysis in 4, and Western blot analysis in 3. Table 1 details their full results.

Analysis

Analytical Focus

The following discussion highlights some of the genes and proteins from articles included in the systematic review. Given that the exhaustive list of genes and proteins (Table 1) we obtained was too large to fully discuss in this review, a selective analytical approach was used to prioritize this discussion of genes. The genes and gene products selected for further discussion were based on articles that demonstrated both gene- and protein-expression differences. Although any type of method was permitted, these findings were most frequently obtained by using RT-PCR and IHC staining. Further consideration was given to genes and proteins that were recurring findings in multiple studies. Also included were microRNAs (miRNAs), important emerging elements of gene and protein expression that are analyzed primarily by microarray and confirmed by using RT-PCR. See Table 2 for a summary of highlighted genes and proteins.
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**Overexpressed Genes in ACTH-PAs**

**hPTTG1**/Securin

Pituitary tumor-transforming gene, or *PTTG*, was first isolated in the GH4 rat pituitary tumor cell line in 1997 by Pei and Melmed.64 Their discovery was based on both differential gene expression and comparison of quantitative mRNA analysis from normal and adenomatous pituitary cells. It was validated by implantation of transfected 3T3 cells into mice, which resulted in tumor formation after 3 weeks.

The human variant, *hPTTG1*, was localized and characterized by Zhang et al.108 and was found to be expressed in normal adult human testis, thymus, colon, small intestine, brain, and lung and in fetal liver. An analysis of PA and various nonpituitary and pituitary carcinoma tissues showed increased *hPTTG1* expression in adenomas and even greater expression in carcinomas. Sáez et al.76 confirmed these findings. From this analysis, *hPTTG1* was classified as an proto-oncogene. As an important aside, some articles used *hPTTG1* and *PTTG* interchangeably; we used whichever name was described in the article.

Additional studies determined the more specific role of *hPTTG1* in PA development. In 1999, Zhang et al.107 showed that increased *hPTTG1* expression was associated with invasiveness in functional PAs. In 2001, Pei63 showed that c-Myc is a downstream target of *PTTG*, and Woloschak et al.108 and Wang et al.95 previously determined by IHC that c-myc was overexpressed in functional ACTH-PAs. Given that c-Myc is also a known oncogene,97 the role of *hPTTG1* in its activation is an important connection. Jallepalli et al.30 showed in 2001 that securin, the protein encoded by *hPTTG1*, is involved in maintaining chromosomal stability during cell division. Furthermore, in 2006, Minematsu et al.53 showed that the expressions of *hPTTG1* and vascular epithelial growth factor (VEGF) were correlated in PAs. In the same year, Filippella et al.22 showed a correlation between *hPTTG1* expression and the Ki-67 nuclear proliferation index, linking *hPTTG1* expression to PA aggressiveness, invasiveness, and recurrence potential. Recent studies more fully elucidated these mechanisms in both pituitary and nonpituitary tissues, representing an active area of research. With the data taken as a whole, the overexpression of *hPTTG1* has a well-supported and well-recognized multifaceted role in PA tumorigenesis.

**NEUROD1**/NeuroD1 (Beta2)

*NEUROD1* is a gene that codes for NeuroD1, also known as Beta2, a basic helix-loop-helix transcription factor involved in tissue determination and differentiation, particularly for neurogenesis75 and the activation of various adult genes (e.g., insulin).48 In 1997, Poulin et al.65 also showed that NeuroD1 is expressed in normal corticotroph cells in the pituitary gland to promote the transcription of proopiomelanocortin (POMC). POMC is the main precur-
<table>
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<th>Authors &amp; Year</th>
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(continued)
TABLE 1. Results of systematic literature review (continued)

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<td>Tagliati et al., 2010</td>
<td><em>Magmas</em> (Tim16)</td>
<td>None</td>
<td>ACTH</td>
<td>RT-PCR, Northern blotting</td>
</tr>
<tr>
<td>Tampanaru-Sarmesiu et al., 1998</td>
<td><em>Tf</em> (transferrin protein)</td>
<td>None</td>
<td>GH, PRL, ACTH, TSH, NF</td>
<td>IHC, ISH</td>
</tr>
<tr>
<td>Tannahill et al., 2002</td>
<td><em>D3, D1</em></td>
<td><em>D2</em></td>
<td>ACTH</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>Theodoropoulou et al., 2004</td>
<td>None</td>
<td><em>MEN-1</em></td>
<td>ACTH, GH, NF, PRL</td>
<td>IHC</td>
</tr>
<tr>
<td>Turner et al., 2000</td>
<td><em>Cyclin A, B, D, &amp; E</em></td>
<td>None</td>
<td>ACTH, GH, PRL, NF</td>
<td>IHC</td>
</tr>
<tr>
<td>Urneoka et al., 2001</td>
<td><em>RCAS-1</em></td>
<td>None</td>
<td>GH, TSH, NF, ACTH</td>
<td>IHC</td>
</tr>
<tr>
<td>Velkeniers et al., 1994</td>
<td><em>IL6</em> (interleukin 6 protein)</td>
<td>None</td>
<td>ACTH, GH</td>
<td>IHC, ISH</td>
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<tr>
<td>Vidal et al., 2003</td>
<td><em>COX2</em></td>
<td>None</td>
<td>General</td>
<td>IHC</td>
</tr>
<tr>
<td>Wang et al., 1996</td>
<td><em>bcl2, c-myc</em></td>
<td>None</td>
<td>ACTH, PRL, GH</td>
<td>IHC</td>
</tr>
<tr>
<td>Wang et al., 2010</td>
<td><em>HMGA1</em></td>
<td>None</td>
<td>NF, GH, GH/PRL, TSH, ACTH</td>
<td>IHC</td>
</tr>
<tr>
<td>Wasko et al., 2005</td>
<td><em>Survivin</em></td>
<td>None</td>
<td>General</td>
<td>RT-PCR</td>
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<tr>
<td>Winczyk &amp; Pawlikowski, 2005</td>
<td>PPAR-γ</td>
<td>None</td>
<td>General</td>
<td>IHC</td>
</tr>
<tr>
<td>Woloschak et al., 1994</td>
<td>c-myc</td>
<td>None</td>
<td>General</td>
<td>IHC</td>
</tr>
<tr>
<td>Woloschak et al., 1996</td>
<td><em>p16</em> (CDKN2A)</td>
<td>None</td>
<td>General</td>
<td>Western blotting</td>
</tr>
<tr>
<td>Xu et al., 2000</td>
<td><em>CRH</em></td>
<td>None</td>
<td>ACTH</td>
<td>ISH</td>
</tr>
<tr>
<td>Yuan et al., 2008</td>
<td>None</td>
<td><em>GSTP1</em> (associated with invasiveness)</td>
<td>GH, PRL, ACTH</td>
<td>IHC</td>
</tr>
<tr>
<td>Zafar et al., 1995</td>
<td>None</td>
<td><em>ER</em> (estrogen receptor protein)</td>
<td>ACTH, GH</td>
<td>RT-PCR, IHC</td>
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<tr>
<td>Zhang et al., 1999</td>
<td><em>PTTG</em></td>
<td>None</td>
<td>NF, GH, PRL, ACTH</td>
<td>RT-PCR</td>
</tr>
</tbody>
</table>

EG-VEGF = endocrine gland–derived vascular endothelial growth factor; FSH = follicle-stimulating hormone; GH = growth hormone; IGF-1 = insulin-like growth factor–1; ISH = in situ hybridization; LH = luteinizing hormone; NF = nuclear factor; PRL = prolactin; TSH = thyroid-stimulating hormone.

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TABLE 2. Summary of highlighted genes and proteins

<table>
<thead>
<tr>
<th>Overexpressed Genes/Proteins</th>
<th>Underexpressed Genes/Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPTTG1/securin</td>
<td>CDKN1B/p27kip1</td>
</tr>
<tr>
<td>NEUROD1/NeuroD1 (Beta2)</td>
<td>CDKN2A/p16</td>
</tr>
<tr>
<td>HSD11B2/11β-hydroxysteroid dehydrogenase 2</td>
<td>KISS1/Kisspeptin</td>
</tr>
<tr>
<td>AKT1/Akt, protein kinase B (PKB)</td>
<td>ACTHR/ACTH-R</td>
</tr>
<tr>
<td>CCND1/cyclin D1</td>
<td>miR-493</td>
</tr>
</tbody>
</table>

The sor molecule for ACTH, which is overproduced by functional ACTH-PAs.

In 2001, Oyama et al. showed that ACTH-PAs overexpress NEUROD1 and its protein product. Fratticci et al. confirmed this result in 2007. This mechanism, however, is not unique to functional ACTH-PAs; it has been shown that NEUROD1 expression levels are similar among ACTH-secreting tumors of both pituitary and nonpituitary origin. Fratticci et al. found that NEUROD1 and its protein product are associated with a significant independent mechanism involved with ACTH overproduction. The role of NEUROD1 in PAs, however, has been the focus of limited research and might represent a meaningful future avenue of exploration.

HSD11B2/11β-Hydroxysteroid Dehydrogenase 2

HSD11B2 is the gene that codes for the enzyme 11β-hydroxysteroid dehydrogenase 2. This enzyme is primarily responsible for oxidizing cortisol into cortisone, which prevents inappropriate saturation of the mineralocorticoid receptors by cortisol, thereby resulting in pseudohyperaldosteronism. Mutations in this enzyme can result in familial syndromes of apparent mineralocorticoid excess.

In 2001, Korbonits et al. demonstrated a significant increase in HSD11B2 transcripts and 11β-hydroxysteroid dehydrogenase 2 staining in functional ACTH-PAs. This finding was paired with concurrent underexpression of HSD11B1 and 11β-hydroxysteroid dehydrogenase 1, which performs the opposite function of converting cortisone to cortisol. Overexpression of HSD11B2 and underexpression of HSD11B1 were confirmed by Rabbitt et al. in 2003, and further analysis showed that the inhibition of 11β-hydroxysteroid dehydrogenase 2 slowed cell proliferation significantly. Although the exact reason remains unknown, Korbonits et al. believed that increased expressions of HSD11B2 and 11β-hydroxysteroid dehydrogenase 2 are related to the reduced negative-feedback effect of cortisol associated with functional ACTH-PAs. However, little additional research has been done to further elucidate and target this mechanism for the treatment of PAs that cause Cushing’s disease.

AKT1/Akt, Protein Kinase B

AKT codes for Akt, or protein kinase B, a serine-threonine kinase that, among other functions, acts as a downstream effector of the phosphoinositide 3-kinase pathway. AKT was originally characterized in 1987 and is a known oncogene. Akt has 3 isoforms (Akt1, Akt2, and Akt3), all of which have similar functions even though their tissue-specific distributions vary. These functions include anti-apoptosis through indirect modulation of phosphatase and tensin homolog (PTEN)-mediated apoptosis, decreased nuclear factor κB degradation, increased p53 regulation, increased cell proliferation by reduced degradation of cyclin D1, and increased cell growth by mammalian target of rapamycin (mTOR) activation. Akt1 and Akt2 are the isoforms most associated with cell proliferation.

In 2005, Musat et al. showed increased expressions of AKT1 and AKT2 with concurrent overexpressions of the Akt1 and Akt2 proteins in PAs compared with those in the normal pituitary gland. This increased Akt activity has been established further in all types of PAs, including non-functional adenomas. Significant research has been performed on this pathway for both pituitary and nonpituitary tumors. Recently, Murasawa et al. demonstrated that pasireotide decreases the activity of Akt in the AtT-20 mouse corticotrophinoma cell line. Liao et al. also showed that miR-200c activates Akt and that blocking it can restore PTEN-mediated apoptosis in the MMQ rat prolactinoma cell line. To date, however, no significant advances in the treatment of human functional ACTH-PAs using Akt as a target have been published.

CCND1/Cyclin D1

The CCND1 gene codes for the cyclin D1 protein, which, along with many other cyclins, functions in the regulation of cyclin-dependent kinases (CDKs). These kinases then go on to activate or inactivate mechanisms most often associated with the cell cycle; for example, cyclin D1 binds to CDK4, which inactivates pRb and allows for movement out of G1 arrest. Overexpression of cyclin D1 is known to be present in a large number of neoplasms, both malignant and nonmalignant, and is one of the most common tumorigenic factors.

Despite the widespread nature of cyclin D1 overexpression, however, little published investigation into its role in human PA formation has been definitively shown until recently. In 2011, Hewedi et al. showed that the expressions of CCND1 and cyclin D1 were elevated in PAs versus those in the normal pituitary gland. These results were confirmed by Lee et al. in 2014 using IHC staining. Both Hewedi et al. and Lee et al. associated relative amounts of cyclin D1 overexpression with recurrence. CCND1 and its protein represent new and potentially fruitful avenues of research for all types of PAs.

Underexpressed Genes in ACTH-PAs

CDKN1B/p27kip1

The CDKN1B gene codes for the p27kip1 protein, which is a member of the Cip/Kip family of CDK inhibitors and is directly involved in regulating CDKs. It is thought that p27kip1 has a direct regulatory role over CDK4 and that cyclin D1 causes sequestration of p27kip1 in the nucleus, which could be one of the factors that allows for overexpressed cyclin D1 to cause cell-cycle derangement despite the regulatory mechanisms. It has been shown that p27kip1 expression inversely correlates with the Ki-67-labeling index and that a loss of p27kip1 expression can result in pituitary hyperplasia and tumorigenesis.

Lloyd et al. showed that PAs consistently show one-eighth to one-half the levels of p27kip1 expression than does
the normal pituitary gland. This result was confirmed in functional ACTH-PAs and carcinomas by Lidhar et al. and in multiple PAs by Bamberger et al. and Komatsubara et al. more recently, Hewedi et al. compared cyclin D1 expression with p27Kip1 expression and found an inverse relationship.

Although research into CDKN1B and p27Kip1 has not been particularly robust, recent publications have pointed to the underexpression of p27Kip1 as a potential cause of multiple endocrine neoplasia type 4 and as a significant co-mutation for the formation of PAs with multiple endocrine neoplasia type 1. However, beyond these studies, no significant work exploring the underexpression of CDKN1B or p27Kip1 as a potential therapeutic target for the treatment of PAs has been published.

**CDKN2A/p16**

CDKN2A is a gene that codes for another CDK inhibitor, p16. Unlike p27Kip1, however, p16 is thought to be more specific to CDK4. As mentioned briefly earlier, the inactivation of CDK4 prevents phosphorylation of Rb, thereby stopping the cell from continuing on in the cell cycle.

In 1996, Woloschak et al. showed attenuated expression of CDKN2A and p16 in multiple types of PAs without any apparent abnormalities in Rb expression. Seemann et al. expanded on this result by showing significant methylation of the CDKN2A locus across all PA subtypes and that dysfunction of p16 was associated with increased PA size. It is interesting to note that Tani et al. showed that CDKN2A expression was roughly 4 times higher in functional ACTH-PAs than in nonfunctioning PAs. They believed that this result explains why functional ACTH-PAs tend to be smaller than other adenoma types.

**KISSIII/Kisspeptin**

KISSI is a metastasis-suppressor gene that codes for kisspeptin. Kisspeptins bind to kisspeptin receptors, G protein-coupled receptors that, when activated, prevent chemotaxis and invasion. These receptors are highly expressed in the pituitary, placenta, pancreas, and spinal cord and are involved in gonadotropin regulation.

Martínez-Fuentes et al. showed that the KISSI gene and/or the kisspeptin receptor gene, KISSIR, were significantly underexpressed in PAs relative to those in the normal pituitary gland. They also showed that the administration of kisspeptin 10 to nonfunctioning and growth hormone–secreting PA cells expressing KISSIR induced apoptosis.

Despite clear foundational evidence that KISSI has a significant role in PAs, most studies have been conducted on its role in the hypothalamic-pituitary-gonadal axis rather than as a target for the treatment of PAs. Given that administration of kisspeptin 10 resulted in apoptosis in cultured adenoma cells, it is quite possible that this mechanism could be harnessed in the future to treat PAs.

**ACTHR/ACTH-R**

ACTHR is the gene that codes for the ACTH receptor protein ACTH-R, a 7-transmembrane G protein–coupled receptor of the melanocortin receptor family. It is commonly known that normal pituitary corticotroph cells have a negative-feedback loop with ACTH and cortisol to prevent overproduction. Morris et al. demonstrated that ACTHR and ACTH-R are underexpressed in ACTH-secreting PAs. As with 11β-hydroxysteroid dehydrogenase 2 overexpression, Morris et al. consider the loss of ACTH-R activity as a potential mechanism for the loss of sensitivity to the normal feedback-inhibition mechanisms that govern ACTH production. No published research has explored ACTHR or ACTH-R expression as a therapeutic target, despite the fact that restoring the functionality of feedback inhibition could, in theory, significantly lower plasma ACTH levels and thus reduce the severity of Cushing’s disease. Morris et al. noted this briefly by demonstrating that functional ACTH-PAs with expressed ACTH-R have lower serum ACTH levels than those that do not. However, no follow-up on this potential target has been done.

**miRNA Expression**

miRNAs are noncoding RNA transcripts that negatively regulate the function of mRNA via binding. They are difficult to classify alongside the genes and proteins examined earlier, because they are solely gene-expression products without measurable translated protein correlates. Several studies have identified overexpression and underexpression of miRNA transcripts via microarray analysis, often with RT-PCR validation. A list of these studies is included in Table 1, although by microarray analysis there are often hundreds of differences in miRNA expression versus that in the normal pituitary gland, so only the most significant differences are reported here for reference.

It should be noted that Stilling et al. found that miR-493 was significantly underexpressed in functional ACTH-PAs compared to that in the normal pituitary gland. miR-493 is a known tumor-suppressor miRNA. Among other targets, miR-493 is thought to regulate the expression of LGALS3, which codes for the galectin 3 protein. Galectin 3 overexpression is found in ACTH-secreting PAs and is associated with tumor aggressiveness. Several miRNA-493 and/or LGALS3/galectin 3 may represent untested, potentially meaningful targets for the treatment of functional ACTH-PAs, especially the aggressive or malignant types.

**Discussion**

**Summary of Key Findings**

Several important genes and proteins were identified by this systematic literature review and included in the subsequent analysis. Of the overexpressed genes identified in functional ACTH-PAs, hPTTGI/secerin, NEUROD1/NeuroD1 (Beta2), HSD11B2/11β-hydroxysteroid dehydrogenase 2, AKT/Akt, protein kinase B, and CCND1/cyclin D1 represent the most well-established candidate genes and collectively form a compelling cross-section of cellular function. Variations in the expressions of these genes and proteins are associated primarily with cell-cycle dysregulation, ACTH overproduction, and reduced cortisol sensitivity. These are all essential elements of what creates the form and function of ACTH-PAs. Figure 2 shows a schematic of the functions of select overexpressed genes and proteins.

Likewise, CDKN1B/p27Kip1, CDKN2A/p16, KISSI/kisspeptin, ACTHR/ACTH-R, and miR-493 represent the same prioritized gene families that were underexpressed...
in ACTH-PAs. These genes and proteins are involved with cell-cycle regulation, tumor suppression, and cortisol-feedback inhibition. A loss of these elements may facilitate downstream gene expression and protein-level effects with significantly reduced regulation. In total, the significant genes and proteins identified in this review represent potential elements for better understanding the behavior of functional ACTH-PAs and for therapeutic targeting of these signature genes/proteins. Figure 3 shows a schematic of the functions of select underexpressed genes and proteins.

**Gene Expression Between Silent and Functional ACTH-PAs**

A thorough discussion of gene expression in functional ACTH-PAs would not be complete without mention of gene-expression differences between silent and functional ACTH-PAs. Although studies that focused primarily on these differences were not analyzed on the basis of our exclusion criteria, we believe that it is important to briefly highlight key differences. Among these differences, galectin 3 is underexpressed in silent versus functional ACTH-PAs, as demonstrated by Jin et al. This finding is consistent with the increased expression of galectin 3 as ACTH-PAs increase in aggressiveness. Tateno et al. performed a more in-depth analysis by exploring the expressions of POMC, NEUROD1, Tpit, CRHR, V1bR, PC1/3, PC2, GR alpha, HSD11B1, HSD11B2, HDAC2, ANXA1, and BRG-1 by RT-PCR. Expressions of POMC, Tpit, CRHR, V1bR, PC1/3, and HSD11B2 were found to be significantly higher in functional ACTH-PAs, and that of NEUROD1 was found to be higher in silent ACTH-PAs.

As these studies show, there exist significant differenc-
es in gene and protein expressions within the continuum of ACTH-PAs. It is important that we not overlook these differences when treating these tumors and exploring new methods and targets.

Limitations

Although a strict systematic review methodology was implemented, significant limitations in this type of review exist. One such limitation is the potential disconnect between gene expression and protein expression. Although the process from gene transcription to functional protein is direct in principle, this process can be altered at many points along the way. In other words, the resulting abnormal protein expression does not necessarily imply a similar direction of gene expression and vice versa. Likewise, what is probed for using IHC only implies the presence of a target epitope rather than any guarantee of functionality or even true protein identity. This is less of a problem with Western blot analysis because of the potential size differences between altered and normal proteins. However, the true nature of what we understand as “expression” is not definitive. Our systematic literature review made no distinction between methods of expression analysis and thus was unprotected from this potential pitfall. In recognition of this limitation, Table 1 provides the methods used in each study.

We attempted to minimize this limitation in our analysis by focusing on studies that definitively showed overexpression or underexpression of both the gene and its corresponding protein. However, the genes and proteins presented are not the only important ones generated by this systematic literature review; we believe that they are a strong representation of the major genes known to be involved in functional ACTH-PA formation and behavior.

We also recognize the limitation of screening for functional ACTH-PAs and that, on the basis of our search criteria, it is impossible to distinguish them from silent ACTH-PAs. Because most cases provided aggregated tumor groups classified by IHC staining without further individual information, it is difficult to say with certainty that our analysis reflects only functional ACTH-PAs that cause Cushing’s disease. However, significant care was taken by the 2 reviewers to include only functional ACTH-PAs, because tumor functionality was one of our inclusion criteria. Because of this care, we believe that the large number of studies performed and included in this analysis provides, on the aggregate, a strong picture of gene and protein expression for functional ACTH-PAs.

Future Avenues of Research

This systematic literature review brings together the many disparate studies done on functional ACTH-PAs over the past 25 years, and we hope to have highlighted potential treatment targets and fundamental gaps in research and understanding that may hold the key to even greater advances.

PAs, although unique in their function and morphology, have much in common with tumors that arise in other areas of the body. However, unlike many other tumor types, they occur frequently, rarely become carcinomas, and are often surgically excised, which leads to an abundance of specimens for analysis. They represent a key platform for not just the treatment of PAs but also for many other solid tumors. It is hoped that greater attention can be paid in the future to these tumors and that breakthroughs in the treatment of them can translate to other tumor types as well.

Conclusions

This systematic review of the literature pertaining to gene-expression variations in ACTH-secreting PAs and those in the normal pituitary gland resulted in a series of candidate genes and proteins that may contribute to tumorigenesis, invasion, and hormone production/secrecion of ACTH. Many of these important genes and gene products have been discovered and characterized without further exploration of their mechanisms and therapeutic value, and they may represent potential candidate genes for future drug or biomarker development. Many of the genes and proteins highlighted here have shared relevance to the treatment of cancers across the body, lending even more urgency and therapeutic value. Further study of all of these genes and proteins could yield a significant breakthrough in our understanding of not just ACTH-PAs but the spectrum of benign tumorigenesis to carcinogenesis, as well.

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Genome and protein expression in pituitary corticotroph adenomas

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