Pituitary gland dysfunction: Clinical and experimental studies

Ph.D. thesis

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Budapest
2015

1. Introduction

Pituitary adenomas (PAs) are common intracranial neoplasms arising from adenohypophysial cells. Their overall estimated prevalence is 16.7% (14.4% in autopsy studies and 22.5% in radiologic studies). Although they are benign tumors, they can lead to increased mortality because of hormone overproduction and compression or invasion of surrounding structures. In addition to genetic events, epigenetic changes, hormonal stimulation, growth factors and environmental agents, abnormal microRNA (miRNA) expression have also been reported to initiate and promote pituitary tumorigenesis. miRNAs are small [approximately 19-25 nucleotides] non-coding RNA molecules involved in the post-transcriptional regulation of gene expression. They have been implicated in many cellular processes, including cell proliferation, apoptosis, cell adhesion and metabolism either as activators – oncomiRs – or inhibitors – tumor suppressor miRNAs – of tumorigenesis. miRNAs have been described to be associated with pituitary tumor type, characteristics (size, invasion) and response to therapy as well as in the regulation of genes associated with the pathogenesis of

PAs. Pheochromocytomas and paragangliomas are benign or malignant tumours of the involuntary nervous system located in the adrenal gland or along the sympathetic or parasympathetic chain. Although both PAs and pheochromocytomas/paragangliomas (pheo/PGL) are relatively rare diseases, they can sometimes occur in the same patient or in the same family.

2. Objectives

The overall aim of the study was to investigate the genetic backround of pituitary tumor formation.

Study I: Patients with germline aryl hydrocarbon receptor-interacting protein (*AIP*) mutations or with a sporadic somatotroph adenoma showing low AIP protein expression have large, invasive and difficult to treat somatotroph adenomas suggesting that low AIP expression is important in determining the pathological characteristics of somatotropinomas. Our objective was to investigate the miRNA regulation of AIP protein in sporadic somatotropinomas.

Study II: PA and pheo/PGL can occur in the same patient or in the same family, and classically they are not part of any multiple endocrine tumor syndrome together. Our aim was to study the possible role of mutations in the genes known to cause pheo/PGL in PA formation.

3. Methods

3.1. Patients

Study I: Thirty-four consecutive somatroph adenomas were included in the study. Exclusion criteria included preoperative somatostatin-analogue therapy, positive family history or features of genetic origin of the PA. Five autopsy normal human pituitaries were used as controls.

Study II: we collected clinical data, genomic DNA, and tumor tissue, where available, from 39 patients (from 27 kindreds) with pheo/PGL and PA in a sporadic (n=19) or familial (n=20) setting. Probands from 23 FIPA families served as controls.

3.2. Genetic studies

Study I: the entire coding sequence of *AIP*, the conserved splice sites and 1200 base pairs of the promoter region were direct sequenced in somatotropinomas. The AIP mRNA expression, the selected miRNA expressions and the endogenous AIP mRNA expression after miR-34a overexpression and inhibition were analyzed by real-time qPCR in somatotropinomas, normal pituitaries or HEK293 and GH3 cell lines.

Study II: sequence analysis of the *AIP*, multiple endocrine neoplasia type 1 gene (*MEN1)*, cyclin-dependent kinase inhibitor 1B gene (*CDKN1B*, coding region and upstream open reading frame) was performed using Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA). Genes implicated in pheo/PGL (MYC-associated factor X (*MAX*), 'rearranged during transfection' tyrosine kinase receptor gene (*RET*), succinate dehydrogenase subunit A (*SDHA*), succinate dehydrogenase complex assembly factor 2 (*SDHAF2*), succinate dehydrogenase subunit B (*SDHB*), succinate dehydrogenase subunit C (*SDHC*), succinate dehydrogenase subunit D (*SDHD*),

transmembrane protein 127 (*TMEM 127*) and von Hippel–Lindau gene (*VHL*) were analyzed using a combination of next generation sequencing, Sanger sequencing and MLPA. In addition, fumarate hydratase (*FH*) was studied in a subset of patients. Loss of heterozygosity study on the *SDHB* locus of the PA and *MEN1* locus of the pheochromocytoma was performed on the available tumor samples.

3.3. Immunohistochemistry

Study I: For semi-quantitative estimate of cytoplasmic AIP immunostaining, slides were scored for pattern [diffuse (score 2) or patchy (score 1)] and for intensity [strong (score 3), moderate (score 2) and weak (score 1)], and the final score was calculated by multiplying the two scores (pattern and intensity), resulting in low (0, 1 or 2) or high (3, 4, or 6) AIP expression.

Study II: Immunostaining for GHRH and MEN1 was performed on relevant pheo samples, while SDHA, SDHB immunostaining was performed on available pituitary tumor samples. Some pituitary samples had

anti-mitochondrial and anti-endoplasmic reticulum lectin 1 (ERLEC1) staining.

3.4. Target site prediction

To identify AIP mRNA-miRNA interaction we initially used algorithms described in the miRNAmap prediction program. The mRNA-miRNA interaction was evaluated by three different criteria: (i) target site predicted by at least two prediction programs, (ii) the target gene contains multiple target sites for the miRNA and (iii) the target sites are located in accessible regions of the RNA as determined by a pre-specified algorithm. We selected to evaluate by qPCR miRNAs that reach all three miRNAmap criteria or at least two miRNAmap criteria and a negative Pearson correlation coefficients for each miRNA and the target gene at least -0.30. Moreover, in order to confirm and to complete our search for miRNAs interacting with *AIP* and to determine the exact miRNA target binding sites in both human and rat *AIP* we utilized TargetScan version 6.2, MicroCosm, FindTar version 3, miRanda and PicTar.

3.5. Cell line studies

Rat GH- and prolactin-secreting PA cell line GH3, the human embryonic kidney cell line HEK293 and the human primary pancreatic adenocarcinoma BXPC3 cell line were used in the studies. A pGL3-*AIP*-3'-UTR vector was generated to examine whether the effect on the luciferase activity (using Dual-Luciferase Reporter Assay System) of the studied miRNAs was specifically due to binding to the predicted binding sites in wild type (WT) or mutated (using QuikChangeXL-site-directed mutagenesis kit) *AIP*-3'-UTR fragment. Cell viability, colony formation and wound-healing assay were used to assess proliferative potential.

3.6. Statistical analysis

The statistical analysis was performed using SPSS version 16.0 for Windows (SPSS, Inc., Chicago, IL, USA) or StatsDirect software (Addison-Wesley-Longman, Cambridge, UK). *P* values < 0.05 were considered statistically significant.

4. Results

4.1. Study I

4.1.1. AIP protein and mRNA correlation

All tumors expressed AIP, with low expression levels (score 1-2) observed in 13 cases (42%). Interestingly, there was no difference in the *AIP* mRNA expression between tumors with low or high AIP protein levels. These data lead us to the hypothesis that post-transcriptional regulation, such as that exerted by miRNAs, may be the cause of the low AIP protein expression.

4.1.2. miRNA expression levels

Based on *in silico* predictions, we selected 11 miRNAs for analysis by real-time qPCR: let-7a, let-7b, miR-202, miR-22, miR-34a, miR-34c, miR-449b, miR-510, miR-612, miR-639 and miR-671. Two miRNAs showed higher expression in tumors with low AIP protein levels compared to tumors with high AIP protein levels: miR-22 and miR-34a.

4.1.3. Correlation of AIP expression, miR-22 and miR-34a levels with tumor invasiveness and response to somatostatin analogues

Eleven out of 13 (85%) somatotropinomas with low AIP protein expression were invasive while 6 out of 18 (33%) somatotropinomas with high AIP expression were invasive (P=0.006). The miR-34a levels did not differ significantly in invasive and in non-invasive somatotropinomas. A total of 26 patients were initiated on long-acting somatostatin analogue (OCT-LAR) after surgery. In 10 patients (38%) acromegaly was considered controlled after OCT-LAR therapy. Only one out of nine patients (11%) whose tumors presented low AIP expression achieved disease control with medical treatment, while nine out of 17 patients (53%) harboring tumors with high AIP expression achieved disease control with OCT-LAR therapy (P=0.045). The miR-34a levels were lower in those patients controlled with OCT-LAR therapy than in the uncontrolled patients.

4.1.4. miR-34a effect on regulation of AIP expression *in vitro*

There are three different predicted target seed regions for miR-34a in the human *AIP*-3'UTR sequence and miR-22 has one binding site, located 42-47 bp downstream of the stop codon of human *AIP*. To verify the *in silico* predicted interaction between miR-34a and miR-22 and *AIP*, we used a pGL3 vector containing the human wild type *AIP*-3'UTR downstream of the coding sequence of Firefly luciferase. Transfection of pre-miR-34a precursor and WT-*AIP*-3'UTR into GH3 cells resulted in a 31±4% reduction of luciferase activity compared with the control scrambled miR (P<0.0001). Transfection of pre-miR-22 precursor and WT- *AIP*-3'UTR into GH3 cells resulted in no reduction of the luciferase activity.

4.1.5. Confirmation of predicted miR-34a binding sites

To investigate which predicted binding site of miR-34a is involved in the miR-34a effect we used deletion mutants targeting the three different bindig sites: MUT_A for the mutated binding site A (c.*6-30), MUT_B for site B and MUT_C for site C. MUT_A leads to a complete loss of miR-34a effect on luciferase activity, while MUT_B and

MUT_C did not change the inhibitory effect of miR-34a on the luciferase assay.

4.1.6. Regulation of endogenous AIP expression by miR-34a *in vitro*

To further characterize the interaction of miR-34a and *AIP in vitro* we measured mRNA and protein levels of endogenous AIP after miR-34a overexpression and inhibition in HEK293 and GH3 cells. Significant decrease in AIP protein level was observed 48h post-transfection with miR-34a compared to scrambled miR control (HEK293 n=7, 17±3%, P=0.001, GH3 n=4, 25±1%, P=0.0005), suggesting that high levels of miR-34a can suppress endogenous AIP protein expression *in vitro*. Although miR-34a overexpression induced a significant decrease in endogenous AIP protein levels, no significant change was seen at the mRNA level in HEK293 and GH3 cells.

4.1.7. The effect of miR-34a on cell proliferation and colony formation

To determine the biological effect of miR-34a overexpression, we investigated its effect on cell proliferation and colony formation in BXPC3 and GH3 cells. Using a cell proliferation assay we observed a significant increase in the number of living cells in culture 24h and 48h post-transfection (13±3% P=0.007, 9±5% P=0.02). In case of the wound-healing assay and colony formation assay the difference were not significant.

4.2. Study II

4.2.1. Genetic screening

Germline alterations were identified in *SDHA, SDHB, SDHC, SDHD, SDHAF2, VHL* and *MEN1* genes in 19 patients with pheo/PGL and/or PAs. Twenty patients had no identifiable mutations in any of the genes tested. None of the patients in our cohort had *AIP* or *CDKN1B* mutations. We identified 11 kindreds (including 16 patients) with germline *SDHX* variants. Seven families had a pathogenic *SDH* mutation, while 4 had a variant of unknown significance. In the PAs, where suitable sample was available, we identified the loss of the wild-type

allele in the adenoma sample compared to the germline DNA. We identified two patients with germline *MEN1* mutation and pheochromocytoma, while all the other tested genes were normal. Both pheochromocytomas showed LOH in the *MEN1* gene.

4.2.2. Pathological features

The PAs of patients with *SDHX* mutations were characterized by intracytoplasmic vacuoles. The histochemical stain periodic acid of Schiff (PAS)/diastase-resistant PAS (DPAS) did not reveal any glycogen accumulation. As *SDHX* mutations are known to alter mitochondrial function, immunostaining was performed for a mitochondrial membrane protein with the anti-113-1 antibody. Vacuoles did not appear to be rimmed by this protein suggesting that vacuolization is not secondary to dilatation of mitochondria. To understand if vacuoles were the result of swelling of the endoplasmic reticulum (ER), we immunostained our samples for the ER marker ERLEC1. None of the vacuoles was lined by this protein indicating that they were not releated to the ER. We used electron

microscopy to further study the nature of vacuoles. Interpretation of ultrastructural features of the tissue retrived from paraffin was limited by suboptimal preservation. The cytoplasm appeared to contain large empty vacuoles unrelated to mitochondria and no obvious membrane were identified to rim vacuoles. Menin staining of the pheochromocytoma samples of patients with *MEN1* mutations showed either no menin positive cells or weakly positive staining nuclei.

5. Conclusion

5.1. Study I

In this study we showed that low AIP protein levels in human sporadic somatotropinomas are associated with high miR-34a expression and that miR-34a can down-regulate AIP protein levels in *in vitro* experiments. In addition, we showed that high miR-34a levels are associated with a lower chance of acromegaly control with SSA therapy and we confirmed our previous findings that low AIP protein expression is associated with a poor response to SSA. Our data demonstrates that the inhibition involves AIP translation repression without

reduction in AIP mRNA, as there was no difference in the AIP mRNA levels between tumors with low or high AIP protein levels. Our findings showed that miR-34a down-regulates AIP protein levels, while miR-22 had no inhibitory effect. Our data suggest that miR-34a reduce *AIP* expression via binding to c.*6-30 at the *AIP*-3'UTR. Endogenous AIP protein expression is inhibited by overexpression of miR-34a *in vitro* in GH3 and HEK293 cells, leading to increased cell viability.

In conclusion, we have demonstrated that miR-34a is overexpressed in sporadic somatotropinomas with low AIP protein levels in the absence of mutations in this gene and that this overexpression is inversely correlated to the response to SSA. Functional studies confirmed that miR-34a down-regulates AIP expression, suggesting the possible involvement of miR-34a in the pathogenesis of sporadic somatotropinomas.

5.2. Study II

Syndromic presentation of PA and pheo/PGL is rare and it is not part of the classical multiple endocrine tumor syndromes. Germline mutations were identified in the

studied PA or pheo/PGL causing genes in 11/27 kindreds with the combination of pheo/PGL and PAs. We show that pituitary adenomas occur in patients with *SDHX* mutations and their pathogensis is likely to depend on the *SDHX* mutation. We also confirm that *MEN1* mutation can predispose to pheochromocytomas. An endocrine rather than genetic causation can link pheochromocytomas secreting hypothalamic releasing hormones (GHRH or CRH) with pituitary changes mimicking the PAs and these cases may present a clinical differential diagnostic problem. In about half of our cases no germline abnormalities were seen, suggesting either the presence of other disease-causing genes or the coincidental occurrence of the pituitary and pheo/PGL tumors. We have identified a novel feature of the PAs of patients harboring *SDHX* variants. The adenoma tissues show extensive vacuolization of cytoplasm. The origin of vacuoles remains unclear. Inactivation of succinate dehydrogenase or VHL can lead to activation of the hypoxia inducible factor (HIF) pathway and a pseudohypoxic state. Indeed, it has been shown increased HIF-1α in an *SDHD* mutated case linked to PA. It is not

known whether the vacuoles seen in the *SDH*-related tumors are due to the pseudohypoxic state, but we did not observe this phenomenon in the *VHL* mutation-related PA. Immunostaining for a mitochondrial membrane protein or for an ER marker did not prove that the vacuoles arise from these organelles.

In conclusion, mutations in the genes known to cause pheo/PGL can rarely be associated with PAs, while mutation in a gene predisposing to PAs (*MEN1*) can be associated with pheo/PGL. Our findings suggest that genetic testing should be considered in all patients or families with the constellation of pheo/PGL and PA.

6. Bibliography of the candidate's publications

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