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Translational research in pituitary tumours

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Abstract

Although effective treatment regimens (surgical resection, drug treatment with dopamine agonists or somatostatin analogues, radiotherapy) have been established for the therapy of most pituitary tumours, a considerable proportion of affected patients cannot completely cured due to incomplete resection or drug resistance. Moreover, even if hormone levels have been normalized, patients with hormone-secreting tumours still show persistent pathophysiological alterations in metabolic, cardiovascular or neuropsychiatric parameters and have an impaired quality of life. In this review reasons for the discrepancy between biochemical cure and incomplete recovery from tumour-associated comorbidities are discussed and the clinical management is delineated exemplarily for patients with acromegaly and Cushing's disease. In view of the development of additional treatment concepts for the treatment of pituitary adenomas we speculate about the relevance of RSUME as a potential target for the development of an anti-angiogenic therapy. Moreover, the role of BMP-4 which stimulates prolactinoma development through the Smad signalling cascade is described and its role as putative drug target for the treatment of prolactinomas is discussed. Regarding the well-known resistance of a part of somatotropinomas to somatostatin analogue treatment, recently identified mechanisms responsible for the drug resistance are summarized and ways to overcome them in future treatment concepts are presented. Concerning novel therapeutic options for patients with Cushing's disease the impact of retinoic acid, which is currently tested in clinical studies, is shown, and the action and putative therapeutic impact of silibinin to resolve glucocorticoid resistance in these patients is critically discussed.

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Invited Author's profile

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Introduction

Pituitary tumours are mostly sporadic monoclonal neoplasias of the different cell types of the anterior pituitary (1). Whereas hormone-inactive pituitary adenomas cause pathophysiological problems (headache, visual field loss, pituitary insufficiency) due to the intracranial mass effects of the expanding tumour, hormone-active adenomas exhibit more complex pathophysiological disorders as a consequence of the excessive hormone production (2) (Fig. 1). For most pituitary tumour types, effective treatment regimens have been established including surgical removal, treatment with drugs such as different dopamine agonists (prolactinomas), the growth hormone receptor antagonist pegvisomant (somatotropinomas) and several somatostatin analogues (somatotropinomas, corticotropinomas), or radiotherapy (2, 3). However, complete resection of critically located or invasively growing adenomas is not possible and a considerable proportion of the tumours are resistant to established drug treatment regimens or alternate radiotherapy. In some cases, surgery or radiation-induced destruction of normal pituitary tissue will lead to partial pituitary insufficiency which then causes additional pathophysiological problems for the affected patients (4) (Fig. 1). Adenomas resistant to standard treatment options are at risk to develop a more aggressive tumour phenotype and in very rare cases will transform to a lethal pituitary carcinoma (1). Moreover, even if the patients with pituitary tumours have been biochemically cured they still show persistent alterations in neuropsychiatric or metabolic comorbidities and have an impaired quality of life (4). Thus, the development of additional treatment concepts still represents a major focus in pituitary tumour research. Using factor-specific or multifactorial (e.g. proteome or transcriptome analyses) approaches, multiple changes in the expression of miRNA, growth factors, cytokines, neuropeptides and/or their corresponding receptors and signalling cascades

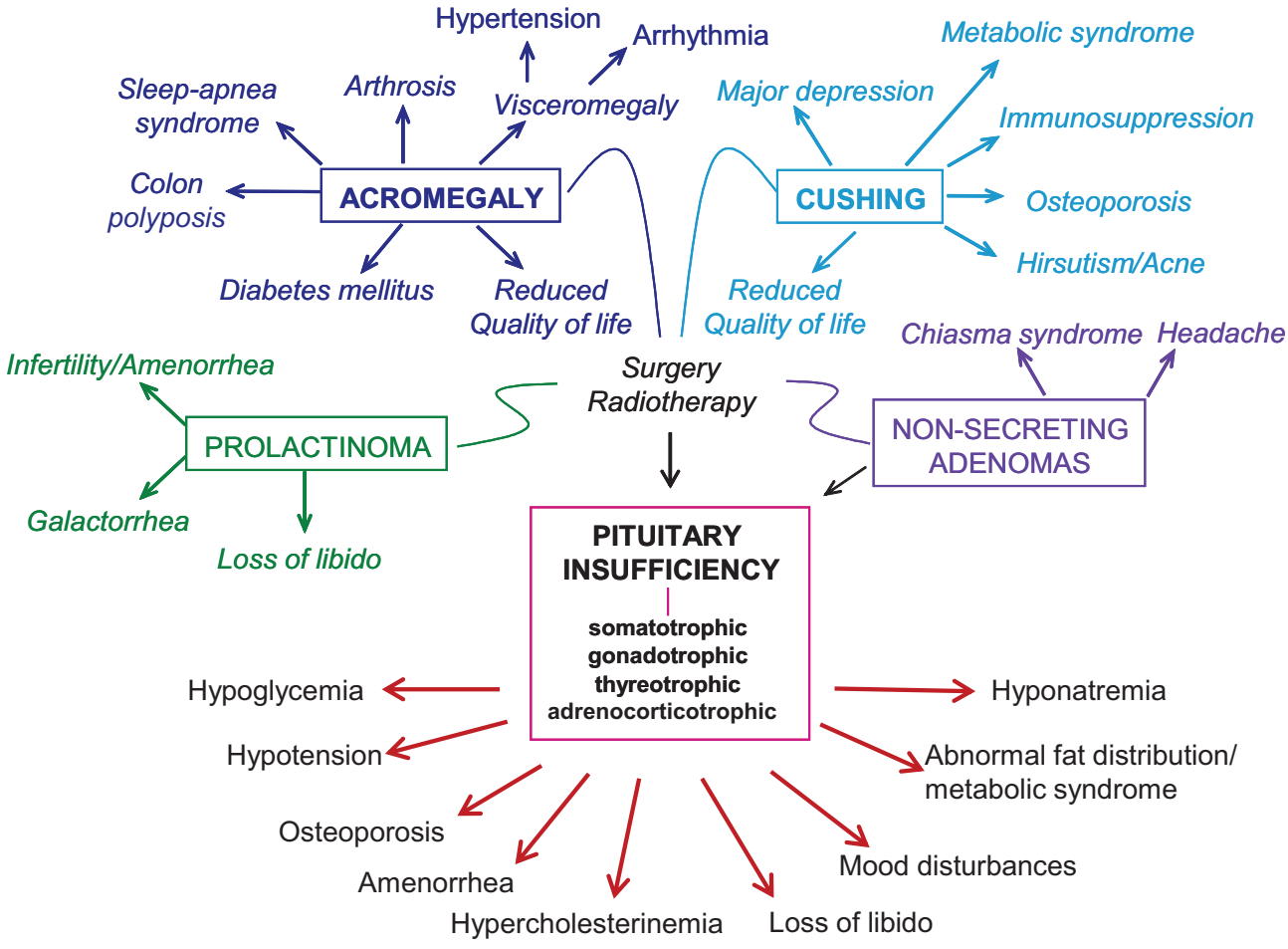

Figure 1
Overview about the comorbidities of pituitary tumours.

have been described in pituitary adenomas in comparison to the normal pituitary (5, 6, 7). Although it is not clear, whether these alterations play a causative role in pituitary tumorigenesis or are a consequence of the neoplastic transformation, they represent putative therapeutic targets to improve the treatment of the adenomas. In this review the discrepancy between biochemical and 'real' cure of patients with pituitary tumours is exemplarily discussed for patients with acromegaly and Cushing's disease. Moreover, the putative role of different factors (Zac1, BMP4, retinoic acid, HSP90, RSUME) as targets of new treatment concepts, some of which are actually approved in clinical trials, are critically discussed.

Clinical management and outcome in Cushing's disease

Cushing's disease (CD), caused by an ACTH-secreting pituitary adenoma, is accounting for 70% of cases of endogenous Cushing's syndrome (CS). The average incidence for CD ranges from 1.2 to 2.4 cases per million per year (8).

Clinical management

Transsphenoidal surgery (TSS) presents the treatment of choice for CD, as it typically leads to rapid and lasting remission of hypercortisolism while preserving the function of the pituitary gland and adrenal glands in most patients. Remission and recurrence rates vary dependent on tumour size, extension, adenoma visibility on MRI, and neurosurgical expertise (9). Patients with persistent or recurrent CD need to be considered for additional therapies such as repeat TSS, radiation therapy, medical therapy or bilateral adrenalectomy in order to minimize consequences of hypercortisolism.

Specialised CD care should not only focus on the early postoperative management, but also on the long-term follow-up. Recently, a structured short- and long-term management plan for CD patients in remission with focus on identifying cognitive impairments and psychiatric disorders, evaluating cardiovascular risk, following pituitary function and detecting possible CD recurrence has been proposed (10).

Somatic and neuropsychiatric comorbidities

CD is associated to increased morbidity and mortality, mostly due to cardiometabolic comorbidities such as arterial hypertension, glucose intolerance, diabetes mellitus, dyslipidemia, thromboembolic complications as well as a hypercoagulable state compared to sex- and age-matched subjects, CD patients present at diagnosis with higher BMI, waist-to-hip ratio, total, low-density lipoprotein cholesterol and total/high-density lipoprotein (HDL) ratio, glucose and insulin, as well as lower HDL cholesterol (11).

Treatment for hypercortisolism has been associated with a significant reduction in mortality and morbidity. However, even after long-term cure of the disease (mean time of hormonal cure, 11 ± 6 years), patients exhibit persistent accumulation of central fat, as in active hypercortisolemia leading to a persistent, unfavourable cardiometabolic risk profile. A recent meta-analysis confirmed that mortality remains increased even after initial biochemical cure, suggesting again that cure does not directly reverse the metabolic consequences of long-term overexposure to cortisol; hypopituitarism, including persistent adrenocortical insufficiency after surgery, may also contribute to the increased mortality risk (12).

Clinical presentation of CD reflects chronic cortisol excess and comprises in more than 70% of patients a broad spectrum of psychological or psychiatric manifestations with (major) depression being the most frequent disturbance. Depression may ultimately even lead to suicide, anxiety, personality changes, mania and psychosis (13). Further neuropsychological disturbances frequently include changes in appetite, insomnia and irritability. Psychic disturbances do not necessarily correlate with the degree of hypercortisolism and can range from mild to severe. Some kind of psychiatric disturbance often remains despite biochemical remission of CD.

As far as personality is concerned, CS patients display significantly higher scores in anxiety, depression and psychotic symptoms compared to healthy controls. In our own series, CD patients reported significantly less novelty-seeking behaviour, presented with more anticipatory worries and pessimism, higher fear of uncertainty, shyness with strangers and appeared to be less extraverted, more neurotic and socially desirable, compared to mentally healthy controls (14).

Several studies point out the impact of chronic exposure to elevated glucocorticoid levels with deficits in cognitive function, concentration and short-term memory as well as brain morphology changes (brain atrophy and low hippocampal volume). Brain volume loss is at least partially reversible following correction of hypercortisolism (15).

It is known that patients with pituitary adenomas frequently suffer from pain syndromes for example, headache due to structural and functional properties of the tumour. In a cross-sectional study, we assessed 278 patients with pituitary disease including 45 CD patients. We recorded a high prevalence of bodily pain (65%) and headache (64%); adrenocorticotropic adenomas were most frequently associated with pain (76%). Headache was equally frequent in patients with macro- and microadenomas ($P=0.266$), while the majority of patients suffered from nociceptive pain (80%) (16).

Quality of Life (QoL)

Impaired quality of life (QoL) in active CD is a stable and consistent finding in many different studies having implications for the long-term management of the disease. The significant impact of chronic exposure to hypercortisolism on patient's health and health-related quality of life (HRQoL) has been demonstrated with generic questionnaires (17). The presence of pain and pain-related disability correlates significantly with impaired QoL in CD (16). All HRQoL parameters improve after treatment with TSS, however, even after long-term remission of CS, impaired QoL persists as a remaining effect of long-standing hypercortisolism, especially in the presence of hypopituitarism (18).

Clinical management and outcome in acromegaly

Not only the clinical management of Cushing's disease represent a major challenge due to a high load of comorbidities. Also in patients with other pituitary diseases such as acromegaly, the long-lasting hormonal excess (in this case GH and IGF-1) leads to severe chronic conditions such as organomegaly, facial disfigurement, arthropathy, diabetes mellitus, hypertension, and neurological and psychiatric complications that reduce patients' quality of life (QoL). Since the complex clinical picture is difficult to ascribe to the correct underlying disease, it often takes up to 10 years of GH/IGF-1 excess until the appropriate diagnostic work-up and treatment is initiated.

Clinical management

The latest consensus on clinical management of acromegaly has been published after the Acromegaly Consensus Conference in April 2017 (3). In brief, clinical management in acromegaly aims at normalizing GH and/or IGF-1 levels to reduce comorbidities, symptoms and mortality rates. After transsphenoidal surgery more than 30% of patients remain biochemically uncontrolled (or cannot be operated in the first place) and are treated with medical therapies (including dopamine agonists, somatostatin analogues and GH receptor antagonists). For those who do not achieve normal IGF-1 levels after second-line therapy, stereotactic radiosurgery remains an alternative.

A multidisciplinary team ideally treating the patient at a pituitary tumour centre of excellence should not only aim at normalizing hormonal levels, but should also diagnose and manage acromegaly-associated comorbidities (3).

Somatic and neuropsychiatric comorbidities

Among the extensive list of symptoms and diseases, metabolic, cardiovascular and respiratory comorbidities seem to be the major causes of elevated mortality rates in acromegaly and should be treated aggressively (3).

To monitor the management of the most severe comorbidities and symptoms, evaluated instruments such as the SAGIT (Signs and symptoms, Associated comorbidities, GH levels, IGF1 levels and Tumour profile), the ACRODAT (Acromegaly Disease Activity Tool) as well as in selected cases the acromegaly-specific patient questionnaire AcroQoL that measures health-related QoL can be considered (3).

Besides the most prevalent metabolic and cardiovascular morbidities, a recent systematic review on neuropsychiatric symptoms underlines once more the clinical relevance of psychiatric morbidities in pituitary disease that are often underdiagnosed and undertreated (19). In acromegaly, neurocognitive alterations were detected in 2–33% of study patients while psychiatric disorders occurred in up to 63% of patients (with depression being the most common).

Although normalized IGF-1 levels achieved by treatment usually improve somatic comorbidities, they may fail to restore mental health problems and normalize patient-related QoL (20, 21). This lack of reactivity to treatment might be due to persistent neuronal changes following chronic GH-IGF-1 excess which could be reflected by grey matter and white matter volume changes, decreased oscillation activity in EEG bands of the prefrontal and middle temporal lobes of the brain and functional neuropsychological alterations (22, 23).

Another reason for reduced mood and QoL in acromegalic patients might be persisting pain syndromes. In the already mentioned study, we assessed 278 patients with pituitary diseases including 81 patients with acromegaly. Patients reported a high prevalence of bodily pain (65%) and headache (64%) correlating significantly with depression and impaired QoL (16).

Quality of life

Besides the reduction of mortality and morbidity, the World Health Organization recognizes the improvement of QoL as the third major treatment goal in chronic diseases. However, QoL often remains reduced relative to the normal population despite long-term biochemical cure in acromegaly.

In a primary study and in a systematic review including 51 studies, we analysed the contribution of various determinants of QoL in several disease stages of acromegaly (24, 25). There was incongruent data to support that biochemical control, or treatment of acromegaly in general, is associated with improved QoL. However, in cross-sectional studies and analyses, factors such as depression and obesity were associated with reduced QoL. This might further support the strategy in which acromegaly patients should be treated with the aim not only to reach biochemical control, but also to consider psychosocial or weight-lowering interventions as additional and independent part of the management.

Expression and function of RSUME in pituitary tumours

RSUME (RWD-domain-containing sumoylation enhancer, also known as RWDD3) was cloned from the pituitary lacto-somatotroph tumour cell GH3 overexpressing the IL-6 cytokine transducer gp130 (26). Five of the seven human RSUME mRNA splice variants that have been described so far, code for different RSUME isoforms and two correspond to non-coding RNAs that suffer nonsense-mediated RNA decay. The five isoforms have an RWD domain in the N-terminus of the protein (26, 27). RSUME was originally found to be highly expressed in various tissues such as pituitary, cerebellum, heart, kidney, pancreas and adrenal gland. RSUME is upregulated by cellular stress such as hypoxia and heat shock (26, 27).

The pituitary tumour cell from which RSUME was cloned showed increased tumorigenic and angiogenic potential when experimentally injected into nude mice with respect to a control GH3 stable cell line. Beside pituitary adenomas, RSUME was found to be expressed in other types of tumours with high angiogenic potential, among them gliomas (28) and pancreatic neuroendocrine tumours (29). Particularly, RSUME is expressed in tissues prone to tumour development in Von Hippel Lindau (VHL) syndrome and is upregulated in several tumours related to this disease (27, 30). Accordingly, it has been associated with decreased survival in a group of renal cell carcinoma patients (30).

Under hypoxic conditions, RSUME expression is upregulated and increases HIF-1and 2α protein levels (26, 30). Consequently, RSUME induces vascular endothelial growth factor (VEGF) mRNA and protein expression during hypoxia (26, 31) (Fig. 2). In line with this, downregulation of endogenous RSUME by shRNA, produces the inhibition of HIF-1α stabilization during hypoxia (26). Interestingly, RSUME sumoylates and has a protein-protein interaction with pVHL in normoxia and negatively regulates the assembly of the ECV (the VHL-E3 ligase complex that produces the degradation of HIF, composed of Cullin2, Rbx1, Elongin B, Elongin C), thus inhibiting HIF-1 and 2α ubiquitination and degradation. Through this mechanism RSUME regulates the loss of function of type 2 VHL mutants (27, 30).

In line with the above mentioned regulation, RSUME expression is increased by hypoxia in pituitary tumours (31). The anterior pituitary is one of the most densely vascularized organs due to the flux of information among the hypothalamus, pituitary and its target organs, which is mediated mainly through hormones transported within the bloodstream.

Hypoxia triggers neovascularization, that is, the formation of new vessels and improves oxygen delivery under persistent hypoxic conditions into the developing tumour through HIF, which induces the expression of different angiogenic factors such as VEGF, bFGF and PDGF. In particular, VEGF, which also stimulates pituitary tumour cell growth, is thought to play a key role in pituitary tumour progression. RSUME has a critical role in this process. As mentioned, RSUME was shown to stabilize HIF-1α during hypoxia, thus inducing expression of angiogenic factors like VEGF (26). Shan *et al*. demonstrated that RSUME mRNA is upregulated in pituitary adenomas and significantly correlated with HIF-1α mRNA levels (31). The relation between RSUME, HIF and VEGF was further proved in pituitary adenomas (32). Hypoxia enhanced RSUME and HIF-1α expression, induced its translocation to the nuclei and stimulated VEGF production both in pituitary tumour cell lines and

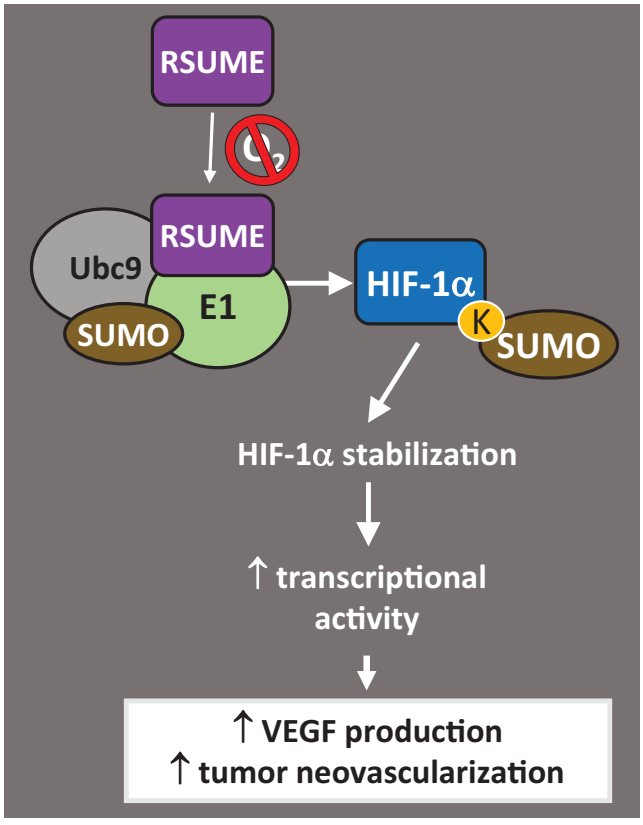

Figure 2
Effect of RSUME in pituitary tumour cells. Under hypoxic conditions, RSUME supports the stabilisation of HIF-1α, and thus, enhances the transcriptional activity of HIF-1 to stimulate VEGF production and pituitary tumour neovascularisation.

human pituitary adenoma cell cultures (31). In addition, when RSUME expression was specifically downregulated, VEGF expression was strongly reduced (31). By supporting pituitary tumour neovascularization, RSUME represents a new putative target for the development of new anti-angiogenic therapeutic treatment for pituitary tumours.

Originally isolated from the rat pituitary tumour cell line GH4, pituitary transforming tumour gene (PTTG) has been shown to be elevated and involved in the growth of pituitary adenomas. No mutations or epigenetic modifications that explain high PTTG expression and action as oncogene have been found. Recently, it has been described that RSUME increases PTTG protein half-life and protein stabilization in pituitary tumour cell lines and enhances PTTG transcription factor and securin activities. RSUME knockdown reverts its actions on PTTG abundance and transcriptional activity and reduces PTTG tumorigenic potential in pituitary cell lines injected in nude mice, accounting for PTTG pathogenic action (33).

In summary, RSUME regulates important pathogenic (i.e. angiogenesis/VEGF and oncogene PTTG) factors and processes that trigger pituitary adenoma development.

Role of BMP-4 in prolactinomas

There has been considerable progress in the understanding of the pathogenic mechanism of prolactinomas in the last decades. For example, oestrogens cause lactotroph hyperplasia and stimulate the expression of vascular endothelial growth factor (34), pituitary tumour-transforming gene (35) and galanin (36). On the other hand, dopamine plays the major role in keeping lactotroph cells suppressed. The dopamine receptor agonist cabergoline is widely used to treat patients with prolactinomas and the dopamine receptor DRD2-knockout mouse spontaneously develops lactotroph hyperplasia and prolactinomas (37). Comparing $DRD2^{-/-}$ tumours versus the normal pituitary by mRNA differential display, we found that the BMP antagonist noggin is downregulated and BMP-4 is overexpressed in prolactinomas (38). BMP-4 is a member of the TGF-beta family of cytokines that plays an important role in cell differentiation and proliferation. The differential expression of BMP-4 and noggin in prolactinomas was then confirmed in prolactinoma models, such as oestradiol-treated rats, and also in biopsy samples from human prolactinomas. Through the Smad pathway, BMP-4 stimulates the expression of the cell cycle regulator c-Myc, resulting in increased cell proliferation. GH3 cells stably transfected with expression vectors that inhibit BMP-4 showed reduced tumourigenicity in nude mice, which supports a stimulatory role for BMP-4 in prolactinoma development *in vivo* (38).

The mechanisms of BMP-4 that impact hormone secretion and gene transcription in the lacto-somatotroph cell line GH3 are known in considerable detail (39) (Fig. 3). We initially demonstrated a crosstalk between BMP-4 and oestrogen in prolactinoma cells which results in a strong additive stimulatory effect at low concentrations on c-Myc expression and cell proliferation (38). This crosstalk occurs also at the level of the transcriptional activity of the prolactin promoter, resulting in an increased prolactin secretion. BMP-4 inhibits the transcriptional activity of oestrogen receptor (ER) at low doses of oestradiol. In contrast, oestrogens stimulate the transcriptional activity of Smad 1/5/8, which is a specific mediator of the BMP pathway. We found that the prolactin promoter contains a specific sequence that is responsible for the response

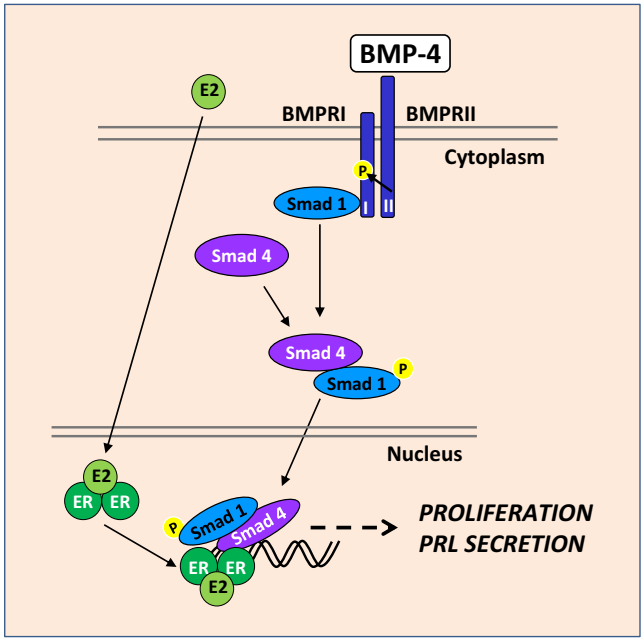

Figure 3

Action of BMP-4 in prolactinomas. After binding to its receptors on the surface of lactotroph tumour cells, BMP-4 induces the phosphorylation of Smad 1 which then forms a complex with Smad 4. After translocation to the nucleus and binding to corresponding specific effector sites the Smad 1/4 complex induces proliferation and prolactin production in lactotroph tumour cells. These processes are enhanced through interaction with the oestrogen (E2)-activated oestrogen receptor (ER).

to the BMP-4 and oestrogen crosstalk. Mutations in the oestrogen response element in the prolactin promoter alone are not enough to inhibit the crosstalk but a Smad1 dominant negative abolishes it. Using serial deletions of the prolactin promoter and CHIP analysis, we were able to identify a sequence responsive to BMP-4 and Smad1. This new response element is located upstream to the transcriptional start site of the prolactin promoter. Smad-4 forms a functional complex with Smad 1/5/8 after BMP-4 stimulation and binds to this DNA sequence, which was demonstrated by chromatin immunoprecipitation, specific mutation, and gel shift assay. The reciprocal regulation mechanism between both pathways, which involves BMP-4, Smads, and ER, contributes to the specific control of prolactin synthesis in prolactinoma cells (40). In addition, both pathways also regulate the mechanisms of cell proliferation that result in the development and progression of prolactinomas. Interestingly, BMP-4 has opposite actions in corticotroph cells as described in detail in the retinoic acid section.

Delineating somatostatin receptor signalling cascades to combat somatostatin analogue resistance in pituitary tumours

With the exception of lactrotroph tumours that are successfully managed with dopamine receptor type 2 agonists, surgery remains the mainstay treatment (2). First-generation somatostatin analogues (SSAs) that target mainly somatostatin receptor 2 (SSTR2) are used for the treatment of acromegaly. However, in 40–60% of patients the SSA treatment fails to achieve biochemical control and/or tumour shrinkage (41). Understanding the mechanisms driving pituitary tumorigenesis may enable us to find ways to combat treatment resistance.

The pathogenesis of pituitary tumours is subject of intense ongoing investigation. Here we will describe our studies on ZAC1, a putative mediator of SSA action in pituitary cells. ZAC1 (Zn finger protein regulating apoptosis and cell cycle arrest, gene name: pleiomorphic adenoma gene-like 1, *PLAGL1*) is a tumour suppressor gene cloned from a corticotroph tumour cell line (42). ZAC1 is downregulated in the majority of pituitary adenomas compared to the normal anterior pituitary, but not in somatotroph tumours from patients that have been treated with SSA preoperatively (43). Using a rodent lacto-somatotroph cell line, we observed that the SSTR2 ligand octreotide upregulates ZAC1 expression and knocking down endogenous ZAC1 abolishes its anti-proliferative action (44). A follow-up immunohistochemical analysis in human somatotroph tumours confirmed the importance of ZAC1 as SSA mediator demonstrating a significant correlation between ZAC1 immunoreactivity and patient response to treatment in terms of biochemical control and tumour shrinkage (45). An independent study confirmed that octreotide upregulates ZAC1, which interacts with the aryl hydrocarbon receptor-interacting protein (AIP) and may be responsible for the SSA resistance seen in acromegaly patients bearing *AIP* mutations (46).

Having ZAC1 as a readout endpoint we embarked in delineating pituitary-specific SSTR2 signalling, that until then was studied mainly in heterologous cell systems overexpressing the receptor (41). We discovered that SSA induces ZAC1 gene expression by suppressing the PI3K/AKT signalling (44). The PI3K/AKT survival pathway is implicated in several aspects of tumour biology including chemotherapy resistance, indicating that its blockage could sensitize pituitary tumour cells to SSA. Indeed overexpressing AKT in our somatotroph tumour cell line abolished the anti-proliferative action of octreotide (47). To test our hypothesis we used as model primary cultures

of human non-functioning pituitary tumours that display high AKT phosphorylation and generally do not respond to SSA treatment despite the presence of SSTRs (48). PI3K pathway inhibition downstream at mTOR level with rapamycin sensitized the resistant tumour to octreotide and improved the response of the few responders as determined by suppression of thymidine incorporation (3H-TdR; surrogate marker of DNA synthesis and cell viability) (47).

Intriguingly, the majority of these NFPA tumours did not respond to the single rapamycin system either, suggesting that the SSA is in turn sensitizing the tumour cells to rapamycin. In fact, the same issue of Cancer Research that published our octreotide signalling data, published a ground-breaking study demonstrating in breast cancer tumours that resistance to the rapamycin analogue everolimus is induced by the inhibitor itself, since it removes a negative feedback loop to AKT that physiologically occurs downstream of mTOR (49). Using a pituitary tumour cell line that does not respond to rapamycin we showed that the upstream inhibitory action of octreotide on the PI3K/AKT pathway renders the rapamycin-induced loss of the negative feedback loop obsolete allowing rapamycin to induce cell cycle arrest (47). A randomized, placebo-controlled, phase 3 study reported improved progression-free survival with the combined everolimus-octreotide-LAR compared to single octreotide-LAR treatment (50). Several clinical trials on the combined treatment with everolimus and SSA have been registered, their majority testing efficacy on advanced neuroendocrine tumours. Despite the initial favourable outcome, latest reports on final overall survival failed to show better efficacy of the combined treatment versus octreotide-LAR (neither did of single everolimus treatment versus placebo) (51). Heterogeneity in the patient population combined with the relatively low patient numbers and severe side effects considering the slow-growing tumours in patients with advanced NET and carcinoid syndrome may account for this discouraging outcome. This setback may give the opportunity to look more closely into other aspects of tumour biology such as tumour heterogeneity and microenvironment and how they influence treatment response from *in vitro* models to patients.

Role of retinoic acid in the treatment of Cushing's disease

Retinoic acid (RA), a derivate of vitamin A, inhibits POMC transcription and its stimulation by CRH in the pituitary ACTH-secreting tumour cell line AtT-20, which is an experimental cellular model of pituitary corticotroph adenomas. This effect was confirmed in primary cultures of human corticotropinomas whereas RA had no effect on non-tumour pituitary cells (52).

In normal pituitary cells, the expression of COUP-TFI might explain why RA does not affect normal ACTH secretion. While normal ACTH-secreting cells express high levels of COUP-TFI, corticotroph tumour cells do not (52). A study in human corticotropinoma samples (29 out of 34) has shown that most of these tumours do not express COUP-TFI (53). In fact, when tumour cells responding to RA are transfected with a COUP-TFI expression vector, the response to RA is abolished (52). This difference between normal and tumour cells makes COUP-TFI expression a promising biomarker of a possible response to RA.

RA works in corticotroph cells through a complex mechanism of action, involving hormone regulation, cell proliferation, differentiation, and chromatin remodelling (Fig. 4). In corticotroph tumour cells, RA induces the expression of the dopamine receptor DRD2 through a process of cell differentiation (54). In addition to the inhibition of hormone secretion, RA inhibits the proliferation of corticotroph and corticosterone-secreting tumour cell lines. These anti-proliferative effects involve transcription factors such as AP-1 and Nur77/Nurr1 (52). In AtT-20 pituitary tumour cells RA also induces the expression of the BMP-4, a cytokine that mediates the anti-proliferative effects of RA (55). Similarly, BMP-4 has been shown to be involved in the inhibitory action of somatostatin analogues on corticotrophs (56). In GH3 and AtT-20 cells, RA-induced expression of BMP-4 involves chromatin remodelling (57). These interactions between RA and BMP-4 in corticotroph cells might provide further insight into the therapeutic potential of RA in corticotroph tumours.

In nude mice bearing AtT-20 xenografts, an *in vivo* model of corticotroph tumours, RA treatment reduces tumour growth and reverses the typical alterations resulting from high levels of glucocorticoids. RA decreases plasma ACTH and cortisol levels, which results in a reduction of adrenal hyperplasia and skin atrophy in these animals (52).

A 6-to-12-month RA treatment regimen in dogs with spontaneous Cushing's disease not only normalized hormonal levels but prevented corticotroph adenomas from recurring. A dose of 2 mg/kg body weight/day of RA results proved to be efficacious and safe, without signs of hepatotoxicity (58). In addition to the reduction of the cortisol/creatinine ratio in urine, RA reduces plasma

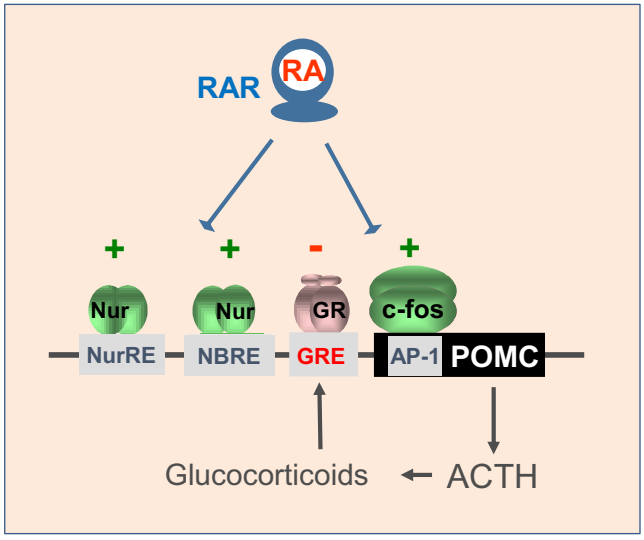

Figure 4

Effect of retinoic acid (RA) in corticotropinomas. The activated RA receptor (RAR) inhibits the transcriptional activity of POMC-stimulating Nur77/Nurr1 and AP-1 transcription factors, and thus, suppresses the excessive ACTH production by corticotroph tumour cells. As these transcription factors also regulate the proliferation of corticotroph tumour cells, RA is also able to suppress the growth of these cells.

ACTH and α-MSH. These hormonal changes lead to the normalization of the oestrous cycle (58). After a period of 6 months of treatment, RA reduced the corticotroph adenoma size, improved clinical signs such as food intake, skin appearance and hair recovery and also strongly prolonged survival (58).

To date, two prospective clinical studies in human patients with Cushing's disease have reported therapeutic effects of RA. RA treatment for a period of 6–12 months normalized ACTH and cortisol levels in many of the treated patients. An initial study of seven patients with Cushing disease (CD) showed that RA administration in doses of 10–80 mg/day reduced significantly UFC levels in five patients and three of them reached normalization (59). Another prospective study of 16 patients with persistent or recurrent CD, in which isotretinoin was given orally in doses ranging from 20 to 80 mg/day for 6–12 months, a response was observed with an initial normalization of the UFC and salivary cortisol in six patients (37.5%). Subsequently, two patients became resistant to the treatment or relapsed. In comparison with non-responders patients, the responding patients had significantly lower mean age and lower mean basal levels of UFC, nocturnal salivary cortisol and ACTH. Therefore, the authors of this study concluded that RA might be more beneficial in patients with mild hypercortisolism (60).

Role of HSP90 in Cushing's disease and potential treatment with silibinin

ACTH biosynthesis and secretion are under the negative feedback control of glucocorticoids in normal corticotroph cells. This mechanism is part of the normal regulation of the response to stress and brings back the hypothalamus-pituitary-adrenal axis to a basal state. In contrast, corticotroph tumour cells in Cushing's disease produce excessive amounts of ACTH despite the high levels of circulating glucocorticoids in these patients. This and the altered response to challenge tests with dexamethasone indicate that the negative glucocorticoid feedback mechanism is impaired in these tumour cells (8). Inactivating mutations of the glucocorticoid receptor (GR) encoded by the nuclear receptor subfamily 3 group C member 1 (NR3C1) gene are rare and have not been found in Cushing's disease in significant numbers. However, other mechanisms that control the activity of the glucocorticoid receptor could explain glucocorticoid resistance in these tumors (61).

In this regard, we discovered a novel biochemical mechanism involving the targeting of Heat Shock Protein 90 (HSP90) (62). HSP90 is highly abundant in basal conditions and its gene expression is transcriptionally induced in response to cellular stress. HSP90 acts as a chaperone that promotes protein folding of hundreds of proteins, many of them involved in disease and cancer progression (63). Among these, are reported kinases that regulate cell cycle and many nuclear receptors, including the glucocorticoid receptor (GR). HSP90 directly interacts with GR ligand-binding domain (LBD), activating it for ligand binding and translocation into the nucleus (64). Therefore, HSP90 plays a central role in the response to glucocorticoids (62).

Depending on its expression level, HSP90 can also impair some of the steps that are necessary for GR function. After becoming fully mature, GR needs to be released from HSP90 (64). However, when HSP90 is overexpressed in corticotroph tumour cells, GR is retained in a cytoplasmic complex, leading to partial glucocorticoid resistance (62). In addition, we did not observe HSP90 overexpression in the normal anterior pituitary therefore our findings might explain the impaired response to glucocorticoids in patients with Cushing's disease (62).

The general structure of HSP90 consists in a homodimer, composed by a highly conserved amino-terminal domain (NTD) connected to a middle domain (MD) and followed by a carboxy-terminal domain (CTD) (64). NTD and CTD are the key players for the chaperone activity of HSP90 and the principal targets of specific inhibitors. In particular, the C-terminal domain is responsible for the final maturation and release of GR from the chaperone complex. C-terminal inhibitors of HSP90 such as novobiocin and the herbal compound silibinin are able to increase the rate of release of mature GR from HSP90 in corticotroph tumour cells, thereby enhancing its transcriptional activity and overcoming glucocorticoid resistance by suppressing proopiomelanocortin (POMC) transcription (62) (Fig. 5). In this way, C-terminal HSP90 inhibitors might be able to compensate HSP90 overexpression and restore the response to glucocorticoids to normal levels. In fact, silibinin administration was able to reverse glucocorticoid resistance in a corticotroph tumour allograft mouse model, leading to a significant reduction in the typical symptoms of the disease. These results in an animal model suggest that silibinin could be potentially efficacious for the treatment of Cushing's disease (62, 65). Silibinin is already in clinical use for other indications and has an excellent safety record, which might facilitate the translation of these results into the clinic for patients with Cushing's disease (66). Clinical studies with a special formulation of silibinin that delivers high levels of the substance into the circulation are now being planned and will soon be launched.

More recently, we showed that HSF1, the transcription factor that controls HSP90 transcription, is also highly expressed and active in Cushing tumours in comparison to the normal pituitary (67). The HSF1 inhibitor KRIBB11 inhibited POMC transcription and ACTH synthesis in the AtT-20 cell line and in primary cultures from Cushing disease patients. As expected, this inhibitory action was mediated by increased GR and suppressed Nur77/Nurr1 and AP-1 transcriptional activities (67). Therefore, HSF1 could be yet another potential drug target for the pharmacological treatment of Cushing's disease.

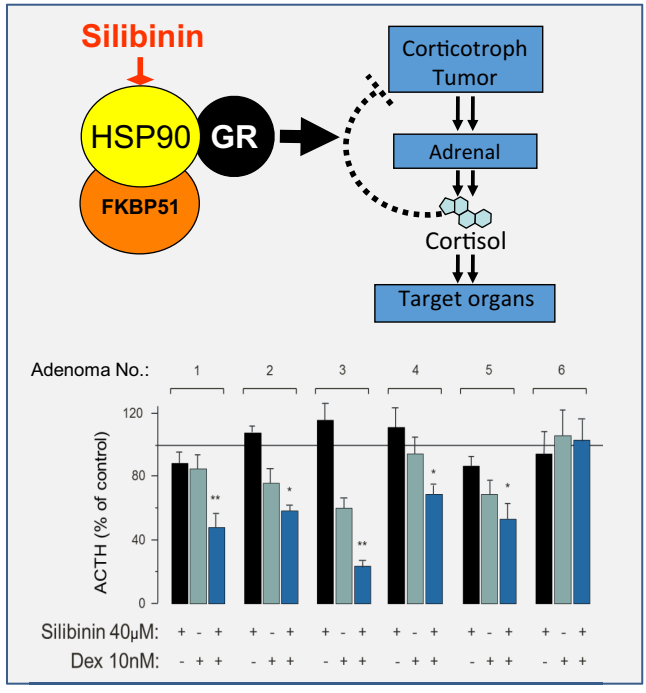

Figure 5
Silibinin-induced recovery of glucocorticoid inhibitory action on ACTH in corticotropinomas. In corticotroph tumour cells, the overexpression of HSP90 leads to a decline of mature glucocorticoid receptor (GR) production and thus to glucocorticoid resistance. Treatment with silibinin, a C-terminal inhibitor of HSP90, leads to an enhanced production of GR and thus the recovery of glucocorticoid response. This is shown in the lower panel, in which the combined treatment of silibinin with the synthetic glucocorticoid dexamethasone (Dex) restores the responsiveness of corticotroph tumour cells to the ACTH inhibitory action of glucocorticoids.

Conclusions

Patients with hormone-active pituitary adenomas still suffer from hormone-excess-associated comorbidities even after the pituitary tumour has been successfully removed and the hormone levels have been normalized. Thus, biochemically cured patients still need a careful postoperative clinical management until they have achieved maximum recovery from tumour-associated health problems such as cardiovascular, metabolic or neuropsychiatric disorders.

For those patients, whose tumours cannot be completely removed and/or who are resistant to established drug treatments, alternative therapeutic options have been shown and discussed in this review. Whereas RSUME and BMP-4 actually remain putative targets waiting for the development of corresponding specific drugs, for other targets clinical trials have already been performed or are under investigation. Based on decoding the interaction of SSTR2, Zac1 and the PI3K/AKT/mTOR pathway an improvement of the inhibitory action of octreotide by the mTOR inhibitor everolimus

was achieved in some patients. RA had not only shown a good control of Cushing's disease *in vivo* in dogs but also in clinical trials with human patients with Cushing's disease. Based on elucidating the role of HSP90 as regulator of glucocorticoid receptor action, a clinical trial has currently been launched that will prove the efficacy of the HSP90 inhibitor silibinin on restoring glucocorticoid response in corticotroph tumours. Thus for patients with distinct types of pituitary tumours some new treatment options will soon be available.

Declaration of interest

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