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Impact of USP8 gene mutations on protein deregulation in Cushing`s disease.

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Precis: We provide a correlation between USP8 mutations and expression of key proteins in corticotroph tumors suggesting these proteins to be USP8 substrates and potential treatment targets against CD.

Abstract

Context Cushing's disease (CD) is a rare disorder with severe sequels and incompletely understood pathogenesis. The underlying corticotroph adenomas harbor frequently somatic mutations in the ubiquitin-specific peptidase 8 (USP8) gene. These mutations render USP8 hyperactive and prevent client proteins from degradation.

Objective To investigate the impact of USP8 mutations on proteins deregulated in CD.

Design 108 pituitary adenomas (75 corticotroph (58 *USP8* wildtype (WT) and 17 USP8 mutated), 14 somatotroph and 19 non-functioning) were investigated by immunohistochemistry. All evaluated proteins (USP8, AVP receptor 1b & 2, CRH receptor, CREB, p27/kip1, cyclin E, HSP90, TR4, EGFR, HDAC2, GR, Cables1) were known to be deregulated in CD. Furthermore, AtT20 cells were transfected with USP8 to investigate the expression of possible downstream proteins by immunoblot.

Results Whereas most of the investigated proteins were not differentially expressed, the cell cycle inhibitor p27 was significantly reduced in USP8 mutated corticotroph adenoma (H-score 2.0±1.0 vs 1.1±1.1 in wt adenomas; p=0.004). In contrast, the chaperone HSP90 was higher expressed (0.5±0.4 vs. 0.2±0.4; p=0.29) and the phosphorylation of the transcription factor CREB was increased in USP8 mutated adenomas (1.30.5±0.40.9 vs. 0.70.5±0.40.7; P=0.014). Accordingly, AtT20 cells transfected with USP8 P720R mutant had higher pCREB levels than WT transfected cells (1.3 ±0.14 vs 1 ±0.23; p=0.13).

Conclusions We could demonstrate that USP8 mutations are associated with deregulation of p27/kip1, HSP90, and phosphorylated CREB. These findings suggest that these proteins are direct or indirect clients of USP8 and could therefore be potential targets for therapeutic approaches in patients with CD.

Introduction

Cushing's disease (CD) is a rare (incidence: 2-4 per million [1]) but life-threatening endocrine disorder caused by adrenocorticotropic hormone (ACTH)-secreting (corticotroph) pituitary adenomas. It is associated with increased morbidity and mortality resulting from chronic exposure to glucocorticoids, secreted by the adrenal cortex in response to ACTH stimulation. Patients with CD often suffer from hypertension, diabetes mellitus, proximal myopathy, and skin atrophy [2]. Accordingly, the majority of deaths associated with CD is caused by cardiovascular, metabolic, and infectious complications. Treatment options include surgical resection of the tumor, irradiation of the sellar region, and medical therapy [3]. However, the available drugs mainly combat the symptoms but not the cause, acting as blockers of the glucocorticoid receptor (GR) (e.g. mifepristone) and inhibitors of either ACTH release (e.g. dopamine agonists, somatostatin analogs) or cortisol secretion (e.g. metyrapone, ketoconazole) [4, 5]. These drugs may have several adverse effects and often also limited efficacy, thus improved pharmacological options are needed (but require an improved understanding of the molecular events causing CD). The definitive treatment of CD is bilateral adrenalectomy which is taken under consideration if other treatments or medication have failed [6]. However, one complication of bilateral adrenalectomy is Nelson's syndrome, an aggressive pituitary tumor that occurs in a small subset of patients [7]. In the last years, important insights in the development of CD have been gained, including the major finding of somatic mutations in the deubiquitinase ubiquitin-specific peptidase 8 (*USP8*) gene in almost half of ACTH-secreting adenomas [8-10]. These mutations were shown to render USP8 hyperactive, resulting in increased recycling of its substrates like the receptor tyrosine kinase (RTK) epidermal growth factor receptor (EGFR), leading ultimately to increased proliferation and increased ACTH secretion [8] bypassing the negative feed-back control exercised through the GR. In addition, other proteins are known to be deregulated in corticotroph tumors like the family of arginine vasopressin receptors (AVPR1b and 2) and the corticotropin releasing hormone receptor (CRHR), both not only involved in ACTH release of the anterior pituitary but also overexpressed in corticotroph tumors [11-15]. Quite recently, also the chaperone heat shock protein 90 (HSP90) and the orphan nuclear receptor 4 (TR4) were shown to be overexpressed in corticotropinomas compared to normal pituitaries [16, 17]. While HSP90 is involved in proper GR folding [16], TR4 induces POMC (pro-opiomelanocortin) promoter transcription [17]. In addition, the histone deacetylase HDAC2 (which deacetylates histone H4 at the POMC promoter, thereby leading to the repression of POMC transcription) was shown to have a reduced expression in a subset of corticotroph tumors [18]. Apart from proteins obviously involved in ACTH hypersecretion, expression or activity of proteins contributing to cell proliferation and tumorigenesis were reported to be modified in corticotroph tumors. These

include the upregulation of cyclin E [19, 20] and EGFR [21, 22] on the one hand, and the downregulation of cyclin-dependent kinase (CDK) inhibitor p27/Kip1 [23-25] on the other. Furthermore, CABLES1, a negative regulator of cell cycle progression is activated in response to glucocorticoids and its expression was shown to be lost in approximately half of corticotroph tumors [26]. Additionally, mutations in the *CABLES1* gene were described as a rare cause for CD [27]. Although a direct link between EGFR signaling and USP8 mutations has been suggested by *in vitro* studies [8, 28], Hayashi and colleagues did not observe activated EGFR signaling in several USP8 mutated tumors with increased levels of POMC mRNA [29] . Thus, it is likely that activated EGFR signaling is not exclusively responsible for increased ACTH secretion in USP8-mutated corticotroph tumors. Other candidates might be the already mentioned deregulated proteins, but also proteins in the cAMP/protein kinase A (PKA) signaling pathway. The latter have been shown to promote ACTH secretion [30], and there is a possibility that increased PKA signaling could be due to deubiquitination through USP8. The aim of this study was to find putative USP8 client proteins that could contribute to the ACTH over-secretion and/or tumorigenesis in CD. For this, we evaluated whether already reported deregulated proteins associated with CD have a significantly altered expression when comparing corticotroph USP8 WT and mutated tumors.

Methods

Tissue collection

In total, we investigated 108 formalin-fixed paraffin-embedded (FFPE) pituitary tumors (corticotroph (n=75), somatotroph (GH-secreting, n=14) and non-functioning (n=19) from individual patients. At the University Hospital Hamburg, 51 corticotroph pituitary adenomas were assembled into one tissue microarray (TMA). In order to choose representative areas with well-preserved morphology of each adenoma, haematoxylin and eosin stained tissues were re-evaluated by one experienced pathologist (WS), marking the corresponding area for tissue punching. Two cores with a diameter of 0.6 mm were punched from each tissue block and arrayed into a new block. Full FFPE slides were used in 24 corticotroph, 12 somatotroph and 19 non-functioning adenomas; these samples had been collected at the University of Wuerzburg. In addition, normal pituitaries (nP) (n=5) were available from autopsies performed at the University Hospital Wuerzburg.

Patients and clinical annotations

For patients with corticotroph adenomas key clinical characteristics are listed in table 1. Diagnosis of CD was performed according to established clinical, biochemical and morphological criteria [31]; the same was true for other pituitary tumor entities. All patients gave written informed consent and the study was approved by the Ethics Committees of the University Hospital Wuerzburg and the Chamber of Physicians in Hamburg.

DNA extraction from FFPE tissue

Full FFPE tissue slides of each 24µm were used to extract DNA, using the QIAamp® DNA FFPE Tissue Kit (Qiagen) according to the manufacturer's instructions. Elution was performed in 30µl H_2O. DNA quality and concentration were determined with the Nanodrop spectrophotometer (Thermo).

Sanger Sequencing

Sanger sequencing was used to determine the USP8 mutation status. Two primers were used, spanning a length of 266 bp around the 14-3-3 binding domain of USP8: USP8fw: ATATGTACCCACCGGAAATG and USP8rev: CAGAGTAGAAACTTTGAAATACAGCA. For amplification of the PCR product in the following reaction, 30-60 ng of DNA was used: 96 °C for 2 min, 10 cycles with 96 °C for 30 sec, 58 °C for 30 sec, and 72 °C for 30 sec. 20 cycles were followed with a reduced annealing temperature of 56 °C. The same primers were used in the sequencing reaction. Results were analyzed with the Genome Genetic Analysis System (ABSciex).

Chromogenic Immunohistochemistry

Slides were deparaffinised in xylol twice before being rehydrated, and antigen retrieval was performed in 10 mM citric acid monohydrate buffer (pH 6.5) within a pressure cooker for 13 min. Endogenous peroxidase was blocked with 3% H_2O_2 in methanol for 10 min, and unspecific binding sites were blocked in 20 % human AB serum at room temperature for 1 hour. Primary antibodies were diluted in PBS and incubated at room temperature for 1 hour. The following antibodies were used: USP8 (Sigma #HPA004869, 1:500), HSP90 (abcam #EPR3953, 1:500), TR4 (Sigma #HPA006313, 1:200), Cyclin E (Santa Cruz #sc-481 1:400), p27/kip1 (BD #610242 1:1000), CREB (cAMP response element-binding protein) (Cell Signaling #9197 1:500), pCREB (Cell Signaling #9198 1:500), CRHR (LSBio #LS-A6687/41992 1:250), AVPR1B (LSBio #LS-A3737/41899 1:250), EGFR (Santa Cruz SC-03 1:500), AVPR2 (LSBio

#LS-B8250/135767 1:250), GR (Cell Signaling #12041 1:400), HDAC2 (abcam #ab32117 1:1600), CABLES1 (abcam #ab75535 1:2000) and N-Universal Negative controls anti-rabbit or anti-mouse (Dako). Signal amplification was performed with Advance HRP Link Kit (Dako) for 40 min and development with DAB+ Liquid Kit (Dako) for 10 min. Nuclei were counterstained with Mayer's hematoxilin for 2 min and blueing was performed for 5 min in running tap water. Stained slides were dehydrated in 100 % EtOH and dried at 56 °C for 30 min before mounting with Entellan.

Semiquantitve analysis of immunoreactivity

Staining intensities were always determined by two independent investigators (I. W. & L. K. or S. S.) and graded 0 (negative), 1 (low), 2 (medium), and 3 (high). The proportion of positive tumor cells was calculated for each specimen and scored 0 if 0 % were positive, 0.1 if 0.1-9 % were positive, 0.5 if 10-49% were positive and 1 if ≥50 % were positive. Cytoplasmic and / or membranous staining and nuclear staining were analyzed independently. A semi-quantitative H-score was then calculated by multiplying the staining intensity grading score with the proportion score as described previously [32]. In case of discrepant results, both investigators jointly assessed the respective stainings, and the final H score was formed by consensus.

Cell culture and transfections

In order to investigate the expression of possible downstream proteins, the murine corticotroph adenoma cell line At-T20 was transfected not only with different USP8 constructs (WT, P720R) but also with empty vector (mock). Cells were seeded into 6 well plates (5x10^5/ well) and transfected with 1µg DNA 24 h later, using Effectene (Qiagen) according to the manufacturer's instructions. USP8 WT and P720R plasmids were donated by Professor Marily Theodoropoulou (Ludwig Maximilian Universität, Munich, Germany) [8] . 48 h post transfection, cells were lysed in RIPA buffer, containing protease and phosphatase inhibitors (Sigma).

Immunoblots

10µg of protein lysates were loaded onto polyacrylamide gradient gels (4-12%, Bio-Rad) and transferred to a PVDF membrane. Antibodies were incubated overnight in 5 % blocking solution and developed on an x-ray film. The following primary antibodies were used: USP8 (Sigma #HPA004869, 1:1000), p-CREB (Cell Signaling #9198 1:500), PKA Cα (BD 5B 1:1000), α-tubulin (housekeeping gene) (Sigma: DM1A 1:20000) and secondary antibodies: goat-α-rabbit-HRP (Jackson ImmunoResearch: 1:10000) and goat-α-mouse-HRP (Jackson ImmunoResearch: 1:10000). Protein-antibody complexes were visualised using the Amersham ECL Prime reagent (GE Healthcare).

Statistical analysis

The Mann-Whitney-U test was performed to determine statistically significant differences between two non-parametric datasets. One-way ANOVA test with Tuckey post-hoc test was done to determine significant differences between different groups of samples. For statistical analyses, GraphPad Prism (version 6.0) was used. P values < 0.05 were considered as statistically significant.

Results

Protein expression pattern in USP8 WT vs USP8 mutated corticotroph adenomas

As already described in the original report [8], cytoplasmatic expression of USP8 was unchanged between USP8 WT and mutated tumors (H-score 1.1±0.6 (WT) vs 1.2±0.5 (mut); p=0.49), but nuclear USP8 was predominant in USP8 mutated tumors (0.7±0.6 (WT) vs 1.6±0.8 (mut); *p=0.0001),* (figure 1 (a) & (b)). EGFR levels were similar in the nucleus (0.6±0.6 (WT) vs 0.6±0.8 (mut) p=0.76), while significantly lower in the membrane in USP8 mutated compared to USP8 WT adenomas (1.3±0.7 (WT) vs 0.9±0.3 (mut) p=0.04), (figure 1 (a) & (c)).

The cell cycle inhibitor p27/kip1 is significantly decreased in USP8-mutated corticotroph tumors

The cyclin-dependent kinase inhibitor p27/kip1 has been shown to be downregulated in corticotroph tumors [21, 23-25]. By analysing USP8 WT and mutated adenomas independently, a reduction in p27/kip1 expression was observed in USP8-mutated adenomas in both the cytoplasm (1±0.7 vs 0.4±0.6; p=0.003) and the nuclei (2±1 vs 1.1±1.1; p=0.004) (figure 1 (a) & (d)). Compared to the other tumor entities, p27/kip1 expression was also only reduced in USP8-mutated but not in USP8 WT tumors (figure 1(d)). Another cell cycle regulator, Cyclin E, has been described as overexpressed in corticotroph tumors [19, 20]. In our cohort, Cyclin E was indeed stronger expressed in all pituitary tumors, independent of their specific hormone secretion compared to the normal pituitary. However, its expression in corticotroph tumors was independent of USP8 mutation status (WT vs mut, cytoplasm: 1.7±0.7 vs 1.5±0.7; p= 0.199, nuclei: 0.8±0.6 vs 0.7±0.5; p=0.533) (figure 1 (a) & (e)).

CREB (cAMP response element-binding protein) is significantly stronger phosphorylated in USP8-mutated than in USP8 WT corticotroph adenomas

It has been demonstrated that the cAMP/PKA signaling pathway (including CREB) is involved in POMC promoter activation [33] and ACTH release from cells of the anterior pituitary gland [30]. We therefore investigated the expression of CREB and its activated form, phospho-CREB, both in USP8 WT and USP8 mutated corticotroph tumors. Since CREB is a transcription factor, both forms were predominantly localised to the nucleus. However, CREB was also weakly expressed in the cytoplasm (figure 1(a) & (f)) whereas phospho-CREB was exclusively nuclear (figure 1(a) & (g)). While CREB protein levels did not differ between USP8 WT and USP8 mutated tumors (neither in the cytoplasm (0.6±0.5 vs 0.4±0.6; p=0.23) nor in the nucleus (2±1 vs 2.1±0.9; p=0.65) (figure 1 (a) & (f))), nuclear levels of phospho-CREB were significantly higher in USP8-mutated adenomas (0.7±0.7 vs 1.3±0.9; p=0.014). Compared to normal pituitaries, nuclear CREB protein expression was higher in all pituitary tumor entities

(figure 1(f)) ($p<0.05$), with similar results for phospho-CREB, even if to a lesser extent (figure 1(g)). Since CREB is phosphorylated by the catalytic subunits of PKA (PKA Cα), we analyzed the expression of the PKA catalytic subunit α in a subset of these tumors (supplementary figure 2 [34]) and found a stronger expression of this subunit in USP8 mutated tumors compared to USP8 WT tumors. Nevertheless, this difference was not statistically significant.

Expression of the orphan nuclear receptor 4 (TR4) expression is not changed between corticotroph USP8 WT and mutated tumors

More recently, orphan transcription factor TR4 (also known as NR2C2) was demonstrated to be significantly overexpressed in corticotroph tumors compared to normal pituitary glands [16, 17]. In our cohort, the expression of TR4 was unchanged between USP8 WT and mutated tumors, both in the cytoplasm (0.8±0.6 vs 0.6±0.6; $p=0.215$) and in the nuclei (2.0±0.8 vs 2.1±1; $p=0.471$) (figure 1 (a) & (h)) or when compared to other pituitary tumors (figure 1(h)).

Further differential protein expression analyses in corticotroph USP8 WT and mutated tumors

We also examined the expression of several markers closely related to corticotroph deregulation in corticotroph adenomas and normal pituitaries. AVPR1b protein levels were extremely low in corticotroph adenomas, but were comparable between USP8 WT and USP8 mutated cells (membranous: 0.07±0.2 (WT) vs 0.08±0.1 (mut); $p=0.166$ and completely absent expression within the nucleus) (figure 2(a) & (b)). In contrast, the 5 normal pituitaries had significantly higher protein levels of AVPR1b in comparison to corticotroph adenomas independent of their mutation status (cytoplasmatic/membranous: 1.8±0.4 vs. 0.07±0.2; $p<0.0001$) (see supplementary figure 1 [34]). Expression of AVPR2 was in general relatively high: its expression varied from H-score 0 to 3 in both USP8 WT and mutated corticotroph tumors (figure 2(a) & (c)). There was no significant difference in AVPR2 expression between USP8 WT and mutated corticotroph adenomas in either cytoplasm (1.5±1 (WT) vs 1.5±1.2 (mut), *p=0.94*) or nucleus (0.1±0.3 (WT) vs 0.1±0.3 (mut), *p=0.66).*

H-scores for CRHR expression were also largely unaffected by the USP8 mutation status, with similar expression levels between USP8 WT and USP8 mutated corticotroph adenomas (membranous: 1.4±0.6 (WT) vs 1.3±0.7 (mut), *p=0.95*) (figure 2(a) & (d)).

When we investigated the expression of the glucocorticoid receptor itself in corticotroph adenomas and normal pituitaries, we did not observe significant differences between USP8 WT and mutated adenomas (figure 2(a) & (e)). Cytoplasmatic staining was very low in corticotroph tumors (0.3±0.5 (WT) vs 0.1±0.3 (mut), *p=0.13),* while nuclei staining was high in most samples (2±0.9 (WT) vs 2±1 (mut), *p=0.9).*

The expression of HDAC2 in corticotroph adenomas was previously shown to be reduced, resulting in glucocorticoid resistance and promoting CD [18]. In our cohort, HDAC2 expression was exclusively nuclear (figure 2(a) & (f)), with only slightly higher expression in USP8 mutated adenomas compared to USP8 WT adenomas (1.6±0.9 (WT) vs 2.1±0.8 (mut), *p=0.09).*

Expression of heat shock protein 90 (HSP90) is increased in USP8-mutated tumors

More recently, HSP90, a chaperone involved in proper glucocorticoid receptor (GR) folding was found to be overexpressed in corticotroph adenomas [16].

To investigate whether this could be due to mutation induced USP8 hyperactivity, we analyzed HSP90 expression. HSP90 was not differentially expressed between USP8 WT and mutated adenomas in the cytoplasm (1.9±0.9 (WT) vs 1.7±1.0 (mut); p=0.448), however, its expression was significantly increased in the nucleus of mutated adenomas (0.2±0.4 (WT) vs 0.5±0.4 (mut); p=0.029) (figure 2(a) & (g)). As observed by Riebold *et al.* [16], HSP90 expression was higher in corticotroph tumors than in normal pituitaries.

Expression of CABLES1 is reduced in USP8-mutated tumors

It has been reported that expression of the negative cell cycle regulator CABLES1 is absent in about half of corticotroph tumours and that CABLES1 expression correlates with p27/kip1 expression [26]. When we analyzed the expression of CABLES1 in corticotroph tumors with known USP8 mutation status, we observed a reduction in CABLES1 expression in mutated adenomas compared to USP8 WT adenomas, both in the cytoplasm and in the nucleus. However, this reduction was only significant in the cytoplasm (*1.1*±0.7 (WT) vs 0.6±0.6 (mut), *p=0.012)* but not in the nucleus (*1.3*±1.1 (WT) vs 0.8±0.9 (mut), *p=0.23)* (figure 2(a) & (h)).

USP8 mutation increases the levels of phosphorylated CREB in the corticotroph adenoma cell-line At-T20

In USP8-mutated adenomas, we did not only observe increased levels of phosphorylated CREB (figure 1(g)) but also a tendency for increased nuclear expression of the PKA catalytic subunit α in USP8 mutated adenomas (supplementary figure 2 [34]). In order to investigate a direct link with USP8 mutations, we transiently transfected the murine corticotroph adenoma cell line At-T20 with USP8 WT, USP8 P720R mutant and empty vector (mock). Cells transfected with both USP8 plasmids showed an increased expression of USP8 (figure 3(a) & (b)). Furthermore, cells transfected with the mutated form of USP8 showed increased levels of phospho-CREB (1.3 ±0.14 vs 1 ±0.23 vs 1,27 ±0.12) and the catalytic subunit α of PKA (1.3 ±0.12 vs 1 ±0.38 vs 1.2 ±0.34) compared to cells transfected with USP8 WT or empty vector (figure 3(a), (c) & (d)).

Discussion

In recent years, two types of molecular alterations have been reported in the context of CD: altered expression of different proteins [2, 35] on one hand, and hotspot mutations in the deubiquitinase gene USP8 on the other (with the latter occurring in around half of the cases) [8, 10, 28]. The underlying molecular causes for the alterations in protein expression, however, have often remained elusive.

As starting point of our study, we hypothesized that activating mutations in USP8 might be responsible for some of these changes. To test this hypothesis, the most relevant of the previously reported deregulated proteins were investigated immunohistochemically. By looking at proteins that are differentially expressed between USP8 WT and USP8 mutated adenomas, which is possibly related to an increased recycling induced by USP8 activating mutations, we aimed to identify potential USP8 client proteins.

The cyclin dependent kinase inhibitor p27/kip1, which is involved in cell cycle arrest [36], has often been demonstrated to be down regulated in corticotroph adenomas [23-25]. In our series, we observed reduced protein expression of p27/kip1 predominantly in USP8 mutated adenomas, which was significantly reduced when compared to USP8 WT tumors. A downregulation of p27/kip1 is likely to result in increased cell cycle progression and probably contributes to cell proliferation. Furthermore, reduced levels of p27/kip1 in corticotroph tumors have been linked to phosphorylated EGFR at the amino acid residue Tyr992 [21] which is able to bind phospholipase C – γ (PLC) [37]. The latter, though, has been shown to facilitate the export of p27/kip1 from the nucleus into the cytoplasm where it is then degraded [38]. In addition, reduced expression of p27/kip1 was shown to correlate with reduced expression of CABLES1, a negative regulator of cell cycle progression that is likely a direct glucocorticoid target [26]. In our cohort, we could confirm this correlation especially with mutated corticotroph adenomas, showing significantly lower CABLES1 expression contributing to glucocorticoid resistance (figure 2). Although the phosphorylation status of EGFR in USP8 mutated cells has not been assessed so far, its expression in USP8 mutated tumors was shown to be increased at least by some groups [8, 28, 29]. In our cohort, EGFR expression was even reduced in USP8 mutated tumours compared to WT, making the theory that USP8 hyperactivity and EGFR recycling directly correlate even more questionable. USP8 mutations seem to be accompanied by reduced levels of p27/kip1, however, the reason for this change remains elusive, as there is no indication that p27/kip is regulated through ubiquitination. One might speculate that another protein is deubiquitinated by USP8, thereby leading to an increased abundance and reduced p27/kip1 levels. To test if USP8 is in close proximity to p27/kip1, we performed a proximity ligation assay in a small subset of these tumors. Only in USP8 mutated adenomas USP8 and p27/kip1 are located in close proximity, possibly in the same complex

but not in USP8 WT adenomas (supplementary figure 3 [34]). A correlation between reduced levels of p27/kip1 and USP8 mutations is therefore likely.

It has been repeatedly demonstrated that mutated USP8 is predominantly located within the nucleus. Consequently, the transcription factor CREB appeared to be a good candidate for direct regulation by USP8. CREB is not only localized in the nucleus, but also phosphorylated by PKA; besides, it is involved in POMC promoter activation [33]. Thus, we investigated CREB in both its unphosphorylated (i.e. inactive) and its phosphorylated (i.e. active) form (figure 1(a), (f) & (g)). With respect to the nucleus, a significant increase of phosphorylated CREB was observed in USP8-mutated tumors compared to USP8 WT tumors (figure 1). It has been outlined that only phosphorylated CREB at serine 133 is able to bind cAMP response elements (CREs) for induction of POMC promoter activation [39]. Since the expression of unphosphorylated CREB was not altered between USP8 WT and mutated adenomas, it can be hypothesized that PKA, which phosphorylates CREB [40], is deregulated. By investigating the expression of the PKA catalytic subunit α (*PRKACA*) in a subset of pituitary adenomas, we found a trend towards increased levels of this protein in USP8 mutated adenomas in the nucleus. However, possibly due to the small sample size (n=21), this discrepancy was statistically not significant. Introduction of the P720R USP8 mutation into At-T20 corticotroph cells increased the levels of phospho-CREB (figure 3(a) & (b)), suggesting that either pCREB or PKA is a direct client protein of USP8. The increased levels of phospho-CREB could lead in turn to enhanced ACTH secretion by activating the transcription of the POMC promoter (figure 4).

The chaperone HSP90 was recently demonstrated to be overexpressed in corticotroph adenomas [16], resulting in increased inhibition of glucocorticoid receptor activity through HSP90-GR binding. Accordingly, GR is no longer able to efficiently inhibit POMC promoter activity and ACTH secretion [41]. In the cytoplasm of corticotroph adenomas, we did not observe differences in HSP90 protein abundance, however, we found significantly higher protein expression of HSP90 in the nuclei of USP8 mutated adenomas (figure 2(a) & (g)). Since mutated USP8 also shows a nuclear localization, one could speculate that HSP90 is a nuclear client protein of USP8 as HSP90 has been already demonstrated to bind GR and is localized in the nucleus [42]. Interestingly, the possible inhibition of GR action by increased HSP90 protein levels in the context of USP8 mutations could be therapeutically relevant. Riebold and colleagues showed that inhibition of HSP90 with silibinin reduced ACTH secretion of At-T20 cells both *in vitro* and *in vivo* [16], making this inhibitor particularly interesting for patients with CD tumors carrying USP8 mutations.

One of the proteins where we observed differences between normal pituitary and corticotroph adenomas was the arginine vasopressin receptor 1b (AVPR1b) which in normal conditions is

activated by vasopressin, thereby potentiating the stimulating effects of CRH on ACTH secretion [43]. AVPR1b is the vasopressin receptor predominantly expressed on corticotroph cells of normal pituitaries and has been shown to be overexpressed in corticotroph adenomas [11-13]. In contrast, we observed a complete loss of the AVPR1b in corticotroph adenomas compared to normal pituitary tissue in our series. This might be due either to the small reference sample size (only 5 normal pituitaries) or (probably more likely) to the fact that our study investigated AVPR1b expression at protein level while all other studies analyzed mRNA expression only [11-13]. The decrease of AVPR1b might be due to increased vasopressin stimulation in corticotroph adenomas compared to normal pituitaries and somatotroph adenomas. We are well aware that this is not in line with the observed responsiveness of corticotroph adenomas /but not of other pituitary tumors) to desmopressin, a synthetic AVP analog that binds all three AVP receptors [13]. Nevertheless, it is well suitable to the observation that AVPR1b may be expressed in primary corticotropinoma cell cultures although the patients did not respond to desmopressin [44]. Of note, both USP8 WT and mutated samples had similarly low levels, indicating that this receptor might not be a direct substrate for USP8. Interestingly, the AVPR2 was much stronger and differentially expressed between different corticotroph tumors, with tumors being characterized by either high or low AVPR2 expression, however, independent of the USP8 mutation status (figure 2(a) & (c)). Since desmopressin also binds this AVP receptor subtype, its high expression at least in some corticotroph tumors could possibly explain the responsiveness of corticotroph tumors to desmopressin. In addition, we observed reduced expression of HDAC2 in corticotroph tumors compared to normal pituitaries (figure 2(a) & (f)) as already described elsewhere [18]. This reduction was not as pronounced in USP8 mutated as in WT tumors, however, we can only speculate that this might be due to USP8 hyperactivity in mutated adenomas.

However, all other proteins that have been demonstrated to be overexpressed in corticotroph adenomas compared to normal pituitaries (including GR, CRHR, cyclin E, and TR4) were unchanged between USP8 WT and mutated adenomas (figure 1 & 2). Although the activating role of CRHR and TR4 in ACTH secretion is well known, both are probably no client proteins of USP8; their overexpression in all corticotroph adenomas may have a different causality instead. For TR4, we also could not corroborate the results generated by Du et al. [17] who described an overexpression in corticotroph adenomas.

Strengths and limitations

To our knowledge, this is the largest study of corticotroph tumors with known USP8 mutation status in which 14 different corticotroph-relevant proteins were investigated. Due to the rarity of the tumor entity, samples had been collected multicentrically over the past decades. However, clinical follow-up of patients was not standardized and potentially interesting clinical

data (e.g. on remission rates or responsiveness to desmopressin) were unfortunately not available.

Conclusion

To summarize, our results demonstrate a possible role of USP8 mutations in the regulation of protein levels for several markers associated with corticotroph adenomas, most notably p27/kip1, CABLES1, HSP90, and activated CREB (figure 4), all of which have already been described as contributors to either ACTH hypersecretion or increased cell proliferation and CD [16]. These findings might have clinical relevance as an array of existing inhibitors could be used for tailoring the best therapy for CD patients with USP8 mutations, especially the HSP90 inhibitor silibinin which is in clinical use and has been to shown to have an excellent safety record [45].

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Table 1: Clinical characteristics of patients with corticotroph adenomas.

	USP8 WT	USP8 mutated	*p value*
Patients n (%)	58 (77)	17 (23)	
Age in years	54 (17-82)	38 (17-77)	*0.02**
Sex n (%)			
Female	36 (62)	17 (100)	
Male	22 (38)	0 (0)	
Maximum adenoma size in mm	10 (4-47)	10 (3-22)	*0.14*
Body mass index in kg / m^2	27 (20-46)	29 (19-46)	*0.14*
Hormone levels prior surgery			
ACTH [pg/ml]bb median (n)	52 ± 106 (38)	68 ± 7 (13)	*0.38*
Cortisol [µg/dl] median (n)	16 ± 2.4 (43)	18 ± 2.6 (12)	*0.11*

Results are given as median (range)

Figure 1: Immunohistochemical investigation of pituitary adenomas (corticotroph, somatotroph and non-functioning) and normal pituitaries. (a) Exemplary staining of an USP8 WT and a mutated (mut) corticotroph adenoma for each protein investigated (scale bars: 100µm). (b) H-score quantification of USP8 expression either in cytoplasm and membrane or in the nucleus. (c) H-score quantification of EGFR expression either in cytoplasm and membrane or in the nucleus. (d) H-score quantification of p27/kip1 expression either in cytoplasm and membrane or in the nucleus. (e) H-score quantification of cyclin E expression either in cytoplasm and membrane or in the nucleus. (f) H-score quantification of CREB expression either in cytoplasm and membrane or in the nucleus. (g) H-score quantification of phospho-CREB expression either in cytoplasm and membrane or in the nucleus. (h) H-score quantification of TR4 expression either in cytoplasm and membrane or in the nucleus. Abbreviations: GH = somatotroph adenomas, NF = non-functioning adenomas, nP = normal pituitary.

Figure 2: Immunohistochemical investigation of corticotroph pituitary adenomas and normal pituitaries. (a) Exemplary staining of an USP8 WT and a mutated (mut) corticotroph adenoma for each protein investigated (scale bars: 100µm). (b) H-score quantification of AVPR1b expression either in cytoplasm and membrane or in the nucleus. (c) H-score quantification of AVPR2 expression either in cytoplasm and membrane or in the nucleus. (d) H-score quantification of CRHR expression either in cytoplasm and membrane or in the nucleus. (e) H-score quantification of GR expression either in cytoplasm and membrane or in the nucleus. (f) H-score quantification of HDAC2 expression either in cytoplasm and membrane or in the nucleus. (g) H-score quantification of HSP90 expression either in cytoplasm and membrane or in the nucleus. (h) H-score quantification of CABLES1 expression either in cytoplasm and membrane or in the nucleus. Abbreviation: nP = normal pituitary.

Figure 3: Western Blot (WB) results of USP8 WT and USP8 P720R mut introduction in At-T20 cells. (a) Exemplary WB of transfected At-T20 cells. (b) Densitometric analysis of WB USP8 bands normalized to loading control (α-tubulin) in comparison to mock transfected cells. (c) Densitometric analysis of WB p-CREB bands normalized to loading control in comparison to mock transfected cells. (d) Densitometric analysis of WB PKA Cα bands normalized to loading control in comparison to mock transfected cells. Analysis of three independent replicates. Overall P ns.

Figure 4: Proposed action of USP8 in corticotroph pituitary adenomas. (a) USP8 WT conditions. To date it has not been ascertained if the three receptors AVPR1b /2 and CRHR are deubiquitinated by USP8 or not. The POMC promoter is normally activated by several transcription factors (including p-CREB) and is inhibited through binding of the glucocorticoid receptor (GR). HDAC2 deacetylates Histone H4 within the POMC promoter, leading to reduced transcription of the *POMC* gene. (b) Mutated USP8 is predominantly located in the nucleus, possibly allowing for targets that are also located within the nucleus. Here, HSP90 or pCREB expression is possibly higher due to USP8 hyperactivity. An increase in pCREB could lead to increased POMC expression and ACTH secretion. Increased HSP90 in the nucleus might be due to increased USP8 activity in the nucleus. This leads to increased HSP90-GR binding and reduced inhibition of the POMC promoter resulting in increased ACTH secretion. Strongly reduced levels of p27/kip1 are associated with USP8 mutations. This observation is also true

for CABLES1, however, neither p27/kip1 nor CABLES1 seem to be direct USP8 client proteins. Grey arrows without further specification symbolize USP8 deubiquitination action; grey inhibitory arrows demonstrate a blockage of normal protein activity; dashed arrow lines mean protein translocation; red arrows symbolize protein upregulation and red crosses symbolize a block in activity.