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Clinical characteristics and surgical outcome in *USP8*-mutated human adrenocorticotropic hormone-secreting pituitary adenomas

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Abstract

Purpose somatic mutations in the ubiquitin-specific protease 8 (*USP8*) gene have recently been described in patients with Cushing's disease (CD). The aim of the study is to verify whether *USP8* mutation may predict early and late outcome of pituitary surgery in patients with CD operated at a single institution.

Methods We performed a retrospective genetic analysis of 92 adrenocorticotropic hormone (ACTH)-secreting pituitary adenomas. Specimens were screened for *USP8* hotspot mutations in the exon 14 with Sanger sequencing. Hormonal and surgical data were compared between *USP8* variant carriers and wild-type tumors.

Results *USP8* variants were detected in 22 adenomas (23.9%) with higher prevalence in women (28.9% vs. 5.3% in men; $p < 0.05$). No significant difference in hormonal levels and tumoral features in relation to *USP8* status was observed. Interestingly, *USP8*-variant carriers were more likely to achieve surgical remission than wild-type adenomas (100% vs. 75.7%; $p = 0.01$). Conversely, recurrence of CD occurred in 23% of *USP8*-mutated patients and in 13% of patients with wild-type adenoma. The recurrence-free survival did not differ significantly between the two groups ($p = 0.42$).

Conclusions ACTH-secreting pituitary adenomas carrying somatic *USP8* mutations are associated with a greater likelihood of surgical remission in patients operated by a single neurosurgeon. Recurrence rates are not related with *USP8*-variant status.

Keywords Pituitary neoplasms · Pituitary surgery · Adrenocorticotropin · Cortisol

Introduction

Cushing's disease (CD) caused by an adrenocorticotropin hormone (ACTH)-secreting pituitary adenoma is a rare but serious condition that may severely impair the quality of life of affected patients and, if untreated, leads to death because of cardiovascular and infectious complications [1]. The first-line treatment of CD is transsphenoidal pituitary surgery aimed at complete resection of the ACTH-secreting adenoma [2]. When performed by a skilled neurosurgeon, pituitary surgery leads to remission of CD in about 70-90% of cases [3–5]. However, these early favorable results are somewhat weakened by late recurrence of disease in 10-20% of cured patients [3, 4, 6, 7].

Pathogenesis of corticotroph adenoma has not yet been fully elucidated. Apart from the very rare occurrence of CD in genetic syndromes, such as multiple endocrine neoplasia type 1, familial isolated pituitary adenoma, and McCune-Albright syndrome [8–11], no consistent somatic mutation had been found in corticotroph adenomas until the very recent discovery of mutations in the 14-3-3 protein binding motif of ubiquitin-specific protease 8 (*USP8;* Ensembl: ENSG00000138592) gene, a deubiquitinating enzyme, in a variable proportion of patients with CD [12–16]. The importance of these mutations in the pathogenesis of CD is probably linked to the regulation of the epidermal growth factor receptor (EGFR) pathway. Indeed, the prevailing view is that mutated USP8 proteins increase deubiquitination of EGFR, which, in turn, leads to increased EGFR

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signaling [12–14], even though other data have challenged this hypothesis [15]. Evidence from *in vitro* studies, suggests that EGFR-mediated pathway has a stimulatory role for the synthesis of proopiomelanocortin (POMC), the precursor of ACTH [17, 18]. Preliminary studies found that patients with *USP8* mutation have a higher mRNA and protein expression levels of somatostatin receptor type 5 than patients with wild-type tumors [15], which suggests a more favorable response to the somatostatin analog pasireotide. This finding suggests that *USP8* mutational status may be involved not only in the pathogenesis of CD but may also determine the response to treatment.

The aim of the present study was to verify whether patients carrying *USP8*-mutated adenomas present different clinical features and surgical outcomes compared with patients with wild-type adenomas in a large cohort of patients with CD operated at a single institution.

Patients and methods

Patients

ACTH-secreting pituitary adenomas from 92 patients submitted to pituitary surgery between 1996 and 2016 at the neurosurgical department of the Istituto Scientifico San Raffaele in Milan, Italy, were available for analysis. Diagnosis of CD was based on classical clinical features and biochemical criteria [19]. All patients had histological confirmation of an ACTH-secreting pituitary adenoma. Demographic, clinical, imaging, and biochemical data before and after surgery were recorded for all patients in a prospectively maintained database.

Hormonal testing before surgery included the corticotropin-releasing hormone (CRH) test in 63 patients, the desmopressin (DDAVP) test in 82 patients, and the high-dose dexamethasone suppression test in 63 patients. Quantitative ACTH response to CRH and DDAVP tests was expressed as percent ACTH peak increase whereas the cortisol response after dexamethasone administration was calculated as the percent cortisol value over baseline. The cut-off to classify a positive response to CRH and DDAVP was set at 30% ACTH increase over baseline and a decrease of cortisol by at least 50% was considered to indicate a positive response to high-dose dexamethasone suppression test.

Remission of CD was defined as hypocortisolism requiring glucocorticoid replacement therapy, normalization of urinary cortisol levels, and suppression of serum cortisol level below 18 ng/mL after an overnight low-dose dexamethasone test, which was routinely performed 5-6 days after surgery. Moreover, remission of CD had to be maintained for at least 6 months after surgery; otherwise, patients were judged surgical failures. Recurrence of CD during follow-up was diagnosed in patients with reappearance of symptoms of hypercortisolism, increased levels of urinary free cortisol, and abnormal suppression of serum cortisol level after low-dose dexamethasone testing. Standard informed consent relating to surgical procedure was obtained from all patients.

USP8 sequencing

As performed by others [14, 15], the presence of *USP8* exon variants was ascertained on RNA. RNA was obtained from formalin-fixed ($n = 50$) or fresh adenoma specimens ($n = 52$); 8 samples were available both fresh and formalin-fixed. Details of RNA extraction from primary cultures and formalin-fixed specimens are provided in our recent publications [20, 21].The presence of corticotroph cells in fresh adenoma specimens was assured by abundant ACTH secretion in primary cultures [22]; as regards formalin-fixed specimens, abundant *POMC* and absent *GH*, *PRL*, *PIT1*, *LHB*, *FSHB* expression was documented by microarray analysis [20]. Samples were subjected to RNA quality control, i.e. reverse-transcription and real-time PCR amplification of ribosomal protein L13A (*RPL13A*) [23], using Taqman probe Hs03043885_g, (Applied Biosystem, Foster City CA, USA). All samples yielded the expected 81 bp transcript at < 30 cycles, attesting to adequate quality RNA.

For amplification of *USP8* exon 14, the most frequent site of mutations reported so far [14], 100 ng RNA was reverse-transcribed (Superscript VILO cDNA synthesis kit; Life Technologies, Carlsbad, CA, USA) with the following oligonucleotide primers: 5' CTTGACCCAATCACTGGAAC 3' (forward) and 5'TTACTGTTGGC TTCCTCTTCTC 3' (reverse) Touch-down PCR was performed using GO TAQ DNA polymerase (Promega, Madison, WI, USA) at 64 °C to 57 °C annealing. PCR products were purified by ExoProStar Illustra enzyme (Ge Healthcare, Chicago, IL, USA) and Sanger sequencing performed using the ABI PRISM Big DYE Terminator V3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on ABI PRISM 3500 analyzer. All chromatographs yielded informative sequencing data. Of note, *USP8* sequence was identical in the 8 samples obtained from both fresh and formalin-fixed specimens (1 adenoma carried the P720R variant, all others wild-type *USP8* sequence)

Statistical analysis

Homogeneity of variance for continuous variables was verified by the Kolmogorov-Smirnov test. For continuous variables with a normal distribution, the mean (± SD) is reported. For variables not normally distributed, the median

Table 1 Clinical characteristics of 92 patients with Cushing's disease according to *USP8* mutational status

Characteristics	All patients ($n = 92$)	WT ($n = 70$)	*USP8*-mutated ($n = 22$)	*P* value*
Age at surgery (years)	38.6 ± 14.0	38.9 ± 14.0	37.4 ± 14.3	0.65
Females, n (%)	73 (79.3%)	52 (74.3%)	21 (95.5%)	0.036
Estimated duration of disease (years)	4.1 ± 3.1	4.0 ± 3.1	4.2 ± 3.1	0.17
Maximal tumor diameter (mm)	11.7 ± 7.9	11.7 ± 8.5	11.6 ± 5.6	0.93
Macroadenoma, n (%)	41 (44.6%)	30 (42.9%)	11 (50.0%)	0.63
Cavernous sinus invasion, n (%)	4 (4.3%)	4 (5.7%)	0 (0%)	0.57
Sphenoid sinus invasion, n (%)	11 (12.0%)	10 (14.3%)	1 (4.5%)	0.29
Surgical remission, n (%)	75 (81.5%)	53 (75.7%)	22 (100%)	0.01

Data are expressed as number, mean ± SD, or percentage

Abbreviations: WT = wild-type. *USP8* = ubiquitin-specific protease 8

and interquartile ranges (IQR) are reported. Student's t test for unpaired data or Mann-Whitney's test were used to compare continuous variables. Chi-squared tabulation or Fisher's exact test were used to compare binomial proportions. Recurrence of CD was assessed by Kaplan-Meier method and differences in subgroups of patients were tested by log-rank test. Patients lost to follow-up were censored at the last follow-up. Adjusted analysis of the factors affecting the risk of recurrence of hypercortisolism was performed with the use of a Cox proportional-hazards regression forward model. Statistical significance was considered at a P value < 0.05 and all reported values are two-sided. All calculations were performed in the statistical software package SPSS, version 20.0 (IBM SPSS Statistics).

Results

Clinical characteristics of patients

The main clinical features of the 92 patients with CD included into the study are summarized in Table 1. Variants in *USP8* were detected in 22 adenomas (23.9%); of note, all variants in the 14-3-3 binding motif of the *USP8* gene have been described previously [12–16]. Age at surgery and estimated duration of disease did not differ between wild-type and *USP8*-mutated patients (Table 1). Five patients (5.6%) were less than 18 years of age at the time of surgery and *USP8* mutation was present in two. As reported by others, *USP8*-variant specimens were clearly more frequent among women than men with CD (28.7% vs. 5.3%, $p = 0.036$). Tumor characteristics were similar in the two groups (Table 1), with comparable tumor size and features of tumor aggressiveness, as indicated by invasion of the cavernous sinus and/or the sphenoid sinus. Interestingly, as reported by Pérez-Rivas and coworker [14], within microadenomas, *USP8*-mutated tumors had a significantly larger maximum tumor diameter (7.5 ± 2.5 mm) than wild-type tumors (6.1 ± 2.0 mm; $p = 0.014$), no significant difference was observed among macroadenomas (15.6 ± 4.9 mm vs. 19.1 ± 8.3 mm for USP8-variant and wild-type tumors, respectively; $p = 0.21$).

Hormonal characteristics

Table 2 summarizes hormonal data in both groups of patients. Basal plasma ACTH and serum cortisol levels were similar in the two groups. No differences in the responses to CRH, both percent ACTH increase and proportion of responders, were observed between patients carrying wild-type and *USP8*-variant adenomas. Further, no significant difference in the proportion of DDAVP responders as well as the percent ACTH increase after DDAVP was observed. Lastly, no significant differences were detected in the response to high-dose dexamethasone suppression test.

Early and late surgical outcome

Overall, 75 out of 92 patients (81.5%) achieved surgical remission. Patients with *USP8*-mutated adenomas had a higher probability of post-surgical remission compared with wild-type adenomas (100% vs. 75.7%; $p = 0.01$). In fact, all 22 *USP8*-mutated adenomas achieved remission after surgery. Of note, the main prognostic determinants of surgical outcome, i.e. tumor size and invasiveness, were similar between the two groups (Table 1).

Recurrence of CD was evaluated in the 75 patients (22 *USP8*-mutated and 53 wild-type) in remission after surgery. The median follow-up in patients carrying *USP8* variants (75 months; IQR, 25 to 173 months) was comparable to wild-type patients (72 months; IQR, 34 to 108 months; $p = 0.61$). No significant difference in postoperative basal cortisol and ACTH levels was observed between patients with *USP8*-mutated and wild-type adenoma (Table 3). Conversely, median length of postoperative hypoadrenalism

Table 2 Hormonal characteristics in 92 patients with Cushing's disease according to *USP8* mutational status

Characteristics	All patients ($n = 92$)	WT ($n = 70$)	*USP8*-mutated ($n = 22$)	*P* value
Basal ACTH (ng/L)	81.1 ± 5.3	83.0 ± 6.7	75.0 ± 5.1	0.52
Basal cortisol (μg/L)	23.7 ± 1.4	24.3 ± 1.8	22.1 ± 1.2	0.49
Positive response to CRH, n (%)	55/63 (87.3%)	46/52 (88.5%)	9/11 (81.8%)	0.62
Peak ACTH after CRH / basal ACTH (%)	383 ± 48	293 ± 67	401 ± 57	0.39
Positive response to DDAVP, n (%)	70/82 (85.4%)	52/61 (85.2%)	18/21 (87.5%)	1.0
Peak ACTH after DDAVP / basal ACTH (%)	340 ± 44	333 ± 53	362 ± 73	0.77
> 50% inhibition after 8-mg DEXA, n (%)	48/63 (76.2%)	38/50 (76.0%)	10/13 (76.9%)	1.0
Nadir cortisol after 8-mg DEXA / basal cortisol (%)	30 ± 4	32 ± 4	23 ± 8	0.29

Data are expressed as number, mean ± SE, or percentage

Abbreviations: WT = wild-type; *USP8* = ubiquitin-specific protease 8; ACTH = adrenocorticotropin; CRH = corticotropin releasing hormone, DDAVP = desmopressin; DEXA = dexamethasone

Table 3 Postoperative clinical characterstics of 75 patients with Cushing's disease in surgical remission according to *USP8* mutational status

Characteristics	Wild type ($n = 53$)	*USP8*-mutated ($n = 22$)	P value
Serum cortisol level (μg/L)	1.9 (1 – 3.8)	1.8 (1.1 – 9.0)	0.50
Plasma ACTH level (ng/L)	8.5 (6 – 16.5)	11.0 (6.7 – 18.2)	0.25
Length of steroid replacement therapy (months)	12 (8 – 24)	6 (3 – 12)	< 0.01

Data are expressed as median (IQR). P values by the U Mann-Whitney test for independent samples

Abbreviations: WT = wild-type; *USP8* = ubiquitin-specific protease 8; ACTH = adrenocorticotropin

requiring glucocorticoid replacement was significantly shorter in *USP8*-mutated than in wild-type patients (6 months, IQR, 3 to12 months, vs. 12 months, IQR, 8 to 24 months; $p < 0.01$).

Recurrence of CD was detected in 12 patients (16.0%). Overall, recurrence-free survival at 5 and 10 years was 83.2% (95% C.I., 72.8; 93.6) and 77.9% (95% C.I., 65.7; 90.1), respectively. Recurrence of CD occurred in 5 of 22 (22.7%) patients carrying *USP8*-mutated adenomas and in 7 of 53 (13.2%) patients with wild-type tumors ($p = 0.31$ by Fisher's exact test). Recurrence-free survival did not differ significantly according to *USP8* mutational status (Fig.1; $p = 0.42$ by log-rank test). The 5-year recurrence-free survival in *USP8*-mutated group was 73.8% (95% C.I., 50.6 - 97.0%) as compared with 88.5% (95% C.I., 78.9 - 98.1%; Fig. 1) in the wild-type group. Multivariate analysis including sex, age at surgery, adenoma size, duration of glucocorticoid replacement, *USP8* status, basal post-surgical ACTH and cortisol levels also failed to demonstrate significant factors predictive of disease recurrence.

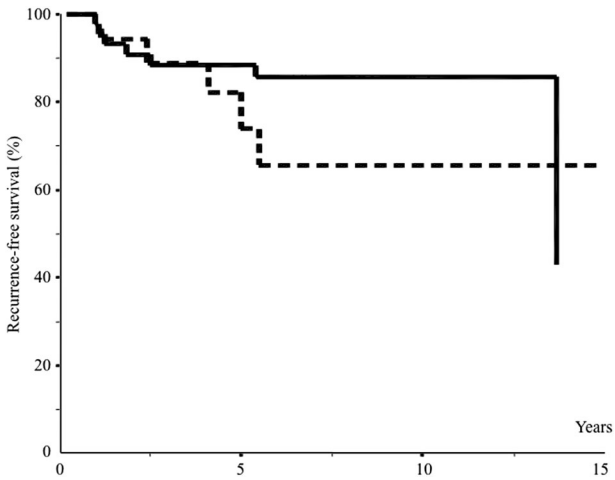

Fig. 1 Kaplan-Meier estimates of recurrence-free survival in 75 patients with post surgical remission of Cushing's disease. Dashed line represents patients with *USP8* mutation ($n = 22$). Continuous line represents patients without *USP8* mutation ($n = 53$). The five-yr recurrence-free survival in patients with *USP8* mutation was 73.8% (95% C.I., 50.6 - 97.0%) as compared with 88.5% (95% C.I., 78.9 - 98.1%; p 0.42 by log-rank test) in patients without *USP8* mutation

Discussion

The present study aims to enhance our understanding of clinical features in patients with CD carrying *USP8*–variants, especially as regards hormonal testing and surgical outcome.

The frequency of *USP8* variants in our 92 patients was 23.9%, which is similar to the 21.4% recently reported by Ballmann and coworkers in a cohort of 42 CD patients investigated using a targeted analysis by next-generation sequencing [24]. Other studies reported a slightly higher frequency of *USP8* mutations, ranging 31.0% to 35.8% [14–16]. In contrast, Ma and coworkers [12] investigated

120 Chinese patients with CD and found *USP8* variants in 75 cases (62.5%). A difference in the ethnic background as well as in recruitment criteria cannot be excluded and only further studies in larger, more varied series of patients with CD may clarify this apparent discrepancy.

Regarding demographic features, we can confirm the marked, significant imbalance in gender frequency, as *USP8*-mutated tumors occurred almost exclusively in women. Similar data have been observed in most [12, 14–16] though not all series [24–26] reported so far, regardless of age, tumor size, and aggressiveness of the corticotroph adenoma. It has been speculated that estrogens may have a growth-stimulating effect on *USP8*-mutated tumoral corticotrophs [14], a hypothesis that is supported by the presence of estrogen receptors on corticotroph cells [27] and the stimulatory effect of estradiol on murine corticotroph cells *in vitro* [28].

In our series, age at surgery did not vary significantly between the carriers of *USP8*-variant or wild-type adenomas, nor did time to diagnosis, in agreement with results from some [12, 15, 24, 25] but not all series [14, 16, 26]. These two latter studies reported younger age among adult *USP8*-variant carriers [14, 26] and older age among pediatric *USP8*-variant carriers [16].

Tumor size and invasiveness were superimposable between *USP8*-variant and wild-type adenomas. Findings on this issue vary, as variant-carrying tumors are reportedly smaller and less invasive in some studies [12, 15]. However, in keeping with other authors [14], we have observed a greater diameter in *USP8*-variant microadenomas compared with *USP*8-wild-type microadenomas. Obviously, case selection comes heavily into play as Hayashi and coworkers [15] intentionally oversampled Crooke's cell adenoma, a rare and aggressive histological variant of ACTH-secreting adenomas while the Chinese series [12] comprised a considerable proportion of invasive tumors (23.5%) and adenomas larger than 4 cm. Of note, among Nelson's adenomas, size of *USP8*-variant was superimposable to *USP8*-wild-type tumors [25]. It should be mentioned that none of the series reported so far reflects the distribution between micro- and macroadenomas usually found in CD, i.e., less than 10-20% macroadenomas, a consequence of the need for adequate pathological specimens to carry out mRNA sequencing.

Information on the characteristics of hormonal responses in *USP8*-variant carriers and wild-type patients is rather scanty. We found no significant difference in the response pattern to both stimulatory, i.e., CRH and DDAVP, and inhibitory, i.e., high-dose dexamethasone, testing. One group of authors had previously reported less cortisol inhibition after 1 mg dexamethasone [13] but greater cortisol inhibition after 8 mg dexamethasone [14], thus some inherent variability may be present. Analysis of basal ACTH secretion shows a marked heterogeneity among different studies. Indeed, plasma ACTH levels were lower in *USP8*-variant carriers than in wild-type patients in one study [15], whereas, in our study, plasma ACTH concentrations were similar in the two groups of patients, as has been observed by others [14, 16, 26]. On the other hand, Ma and coworkers [12] found that basal ACTH secretion was higher in *USP8*-variant than in wild-type adenomas when hormone levels were normalized for tumor size, leading the authors to hypothesize that *USP8*-mutated corticotroph cells possess a greater capacity to produce ACTH [12].

An important finding of our study is the higher remission rate in *USP8*-mutated group as compared with wild-type group. Indeed, all *USP8*-variant adenomas achieved surgical remission. Similar data in ACTH-secreting *USP8*-variant adenomas has been reported by one series [15], whereas other multicenter studies reported superimposable remission rates [12, 14, 26]. Our study is notable in this regard because the main characteristics associated with surgical outcome, namely, tumor size and tumor invasiveness into the cavernous sinus, were well balanced between the two study groups, thus eliminating a potential bias in the interpretation of early surgical outcome. In a previous multicenter study, Pérez and coworkers [14] reported lower rates of postsurgical adrenal insufficiency in *USP8*-variant carriers, without however distinguishing according to surgical outcomes; thus, it is unclear whether remission rates differed in this series. Interestingly, we also observed shorter requirements for steroid replacement therapy in *USP8*-variant carriers in remission, compared with *USP8*-wild-type patients, although postsurgical cortisol and ACTH levels were superimposable among the two groups of patients in remission.

USP8 mutational status might also be linked to the risk of disease recurrence. The large Chinese series [12] reported equal recurrence rates but shorter mean recurrence period in *USP8*-variant carriers than in wild-type patients. Faucz and coworkers reported that, among pediatric patients, recurrences were more frequent in *USP8*-variant carriers [16], even though follow-up was very short (median 17 months). Very recently, Albani and coworkers [26] showed a significantly higher risk of recurrence of disease after initial remission in *USP8*-variant carriers than in wild-type tumors (58% vs. 18%). In this study, the length of follow-up was adequate with 34 of the 48 patients followed for at least 10 years. However, the very high recurrence rate in *USP8* mutated patients is rather unusual and surgical procedures were performed in different neurosurgical centers, thus adding a possible confounder. In our experience, *USP8* mutational status was not predictive of the risk of disease recurrence. Our analysis included a higher number of patients compared with other series and the overall recurrence rate in our patients (16%) seems representative of that

reported in the literature [3–5, 7]. In this context, survey of long-term outcomes in patients with Nelson's syndrome did not reveal differences regarding remission and survival [25]. The contrasting results depicted above may well be explained by the low absolute number of patients with recurrence of Cushing's disease in this study as well as in the above mentioned studies, which lowers the statistical power of the analysis.

Our study has some drawbacks, as with all retrospective studies. Further, the requirement for adequate tissue samples inevitably excluded minute corticotroph adenomas, thus skewed - in our as well as in others' series - prevalence of micro- and macroadenomas. On the other hand, this is a single center study, with a homogeneous approach to patients' data collection and treatment evaluations. Moreover, all surgical procedures were performed by the same, experienced neurosurgeon (PM), which is one of the most important factor affecting early and late surgical outcome of pituitary adenomas [29], thus eliminating the unavoidable bias of different surgeons performing the surgical procedure, typical of multicenter studies.

Conclusion

Our study shows that patients carrying corticotroph adenoma *USP8* variants are more likely to undergo surgical remission, once the confounding effect of different neurosurgeons performing surgical removal of the ACTH-secreting pituitary adenoma has been removed. Conversely, in the same study context, risk of long-term recurrence is not linked to *USP8* mutational status.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For this type of study formal consent is not required.

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