Somatic *USP8* mutations are frequent events in corticotroph tumor progression causing Nelson's tumor

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Abstract

Objective: Somatic mutations in the ubiquitin-specific protease 8 (*USP8*) gene are frequent in corticotroph tumors causing Cushing's disease (CD). Corticotroph tumor progression, the so-called Nelson's syndrome (NS), is a potentially life-threatening complication of bilateral adrenalectomy in patients with refractory CD that is caused by the development of an ACTH-secreting tumor of the pituitary gland. Whether *USP8* alterations are also present in progressive Nelson's tumors has not been studied in detail so far.

Design and Methods: Retrospective, multicenter study involving tumors from 33 patients with progressive corticotroph tumors (29 females) and screening for somatic mutations on the mutational hotspot of the *USP8* gene in the exon 14 with Sanger sequencing.

Results: Fifteen out of 33 tumors (45%) presented with a mutation in the exon 14 of *USP8*, with c.2159C>A (p.Pro720Gln) being the most frequent (9/33), followed by c.2155_2157delTCC (p.Ser718del, 4/33) and c.2152T>C (p.Ser718Pro, 2/33). This prevalence is similar to that previously reported for CD. Mutations were found exclusively in females. Other variables, such as age at diagnosis with NS, body mass index, hyperpigmentation, visual field defects, adenoma size or mortality, did not significantly differ between patients with wild-type and mutant tumors. Patients with *USP8* mutant tumors exhibited higher levels of plasma ACTH after surgery (median: 640 vs 112 pg/mL, $P=0.03$). No differences were observed in ACTH normalization (<50 pg/mL) and tumor control after surgery for Nelson's tumor.

Conclusion: Somatic mutations in *USP8* are common in Nelson's tumors, indicating that they do not drive the corticotroph tumor progression that leads to NS, and may be associated with a less favorable biochemical outcome after surgery for Nelson's tumor.

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Introduction

Bilateral adrenalectomy is an effective and relatively safe procedure to control hypercortisolism in patients with Cushing's disease (CD) when surgery and other therapies failed or are not applicable (1). In addition to a lifelong hormone replacement, patients need an extensive follow-up due to the risk of developing corticotroph tumor progression also known as Nelson's syndrome (NS). NS is a potentially life-threatening complication caused by the growth of a functional corticotroph tumor after bilateral adrenalectomy, which is generally considered a progression of the initial corticotroph adenoma (2). Tumors causing NS can be aggressive as well as refractory to different therapies and, in some cases, can evolve to pituitary carcinomas (3, 4).

Frequent clinical manifestations are cutaneous hyperpigmentation due to an excess of pro-opiomelanocortin derivatives, as well as headache, visual defects and cranial nerve palsy due to tumor enlargement and compression. However, not all symptoms are presented in each NS case (5, 6) and thus there is a lack of consensus both on the definition and the diagnostic criteria of NS (5, 7, 8, 9). Of note, it has also been suggested to replace NS by the term corticotroph tumor progression since incident tumor growth can be identified in up to 50% of the patients after modern MRI imaging years before hyperpigmentation develops (7).

The prevalence of the syndrome is variable, ranging between 8 and 29% of CD patients that undergo bilateral adrenalectomy. Furthermore, it presents a wide time interval in its onset, from few months to more than 10 years after adrenalectomy (2). Accordingly, a lifelong close follow-up is recommended for early detection of NS (7, 10), thereby pointing to the need of disease and outcome markers as well as new molecular candidates for targeted therapies. It is not clear whether corticotroph tumor progression is the consequence of a more aggressive tumor behavior or the complete loss of glucocorticoid feedback after bilateral adrenalectomy. To date, only rare somatic alterations have been described in Nelson's tumors (11, 12) and there is a lack of information about the molecular processes underlying the development of the syndrome.

Recently, the gene coding for the ubiquitin-specific protease 8 (*USP8*) has been identified to play a remarkable role in the pathogenesis of CD. *USP8* is mutated in approximately 50% of pituitary adenomas causing CD (13). So far, all *USP8* mutations in CD tumors are located in a single mutational hotspot in exon 14 (14, 15, 16, 17). The resulting *USP8* mutants show higher activity than the

wild-type and trigger ACTH secretion in pituitary cells through EGFR-dependent signaling (17). It is noteworthy that *USP8* mutations represent an exclusive trait of CD and are not detected in any other type of pituitary tumors nor in ACTH-producing tumors of extrapituitary origin (15, 16, 17, 18, 19, 20). The aim of this study is to investigate the mutational status of *USP8* in tumors of patients diagnosed with NS.

Subjects and methods

Patients and samples

This study was approved by the Ethics Committee of each participating institution and in compliance with the Declaration of Helsinki. All patients gave written informed consent after full explanation of the purpose and nature of all procedures used. We assembled a cohort of 41 tumor samples (33 Nelson's tumors and 8 original CD) originating from 33 patients treated in 7 participating centers in Europe and Brazil. Patients were included in our study according to the definition of NS given by Barber *et al.* (8): diagnosis of CD and, after undergoing bilateral adrenalectomy, development of a pituitary mass expanding in size and/or an increase of the levels of plasma ACTH > 500 pg/mL with progressive elevations of >30% on at least three consecutive occasions. When available, we also studied the initial CD tumor in addition to the Nelson tumor ($n=8$). Basal ACTH was defined as the levels of plasma ACTH at the time of diagnosis of CD. Pre- and postoperative measurements referred only to the surgery in which Nelson's tumor was collected, at least 20 h after the last administration of glucocorticoid. ACTH normalization was defined as postoperative plasma levels <50 pg/mL. Tumor control was defined as lack of adenoma growth and absence of recurrence.

DNA extraction and sequencing

Genomic DNA was extracted from 20 fresh-frozen and 21 FFPE corticotroph tumors using the Maxwell Tissue DNA Kit (Promega) or the FFPE DNA mini kit (Qiagen), respectively. The DNA sequence corresponding to the exon 14 of *USP8* was amplified with the GoTaq DNA polymerase (Promega) and sequenced by means of Sanger sequencing as described previously (14). The chromatograms were analyzed using the Mutation Surveyor software v4.0.9 (Soft Genetics).

Statistical analysis

Statistical analysis was performed with the software package SPSS v23 (IBM). Nominal variables were compared using χ^2 or Fisher exact test when needed. Mann–Whitney *U* test and Kruskal–Wallis test were used for the analysis of non-parametric variables, such as age, time intervals and plasma levels of ACTH. Wilcoxon signed ranks and Friedman test were used to study ACTH variations over time in wild-type and mutant groups separately. Correction for multiple comparisons was performed by means of the Dunn–Bonferroni test. A *t*-test was used to assess the effects of mutations on tumor size and body mass index. Categorical variables were analyzed using a Chi-square test. An exact, two-tailed significance level of $P < 0.05$ was considered to be statistically significant.

Results

To investigate the presence of *USP8* mutations in tumors of patients with NS, we sequenced the exon 14 of *USP8* in tumors from a cohort of 33 patients, 4 males and 29 females. Fifteen cases have been reported elsewhere (3, 12, 21). Except for one case, all patients in our series presented with hyperpigmentation. Transsphenoidal surgery was the treatment of choice for CD in 20 patients, whereas 12 underwent bilateral adrenalectomy without any previous treatment.

In 15 out of 33 patients (45.5%), we found somatic *USP8* mutations in Nelson's tumors. This proportion is similar to that reported for CD (47.3%; Fig. 1) (14, 15, 16, 17). The substitution c.2159C>A (p.Pro720Arg) was the most frequent alteration (9/33 cases, 27.3%). Other mutations were c.2155_2157delTCC (p.Ser718del) in 4/33 cases (12.1%) and c.2152T>C (p.Ser718Pro) in 2/33 cases (6.1%). We could obtain the original tumor prior to the bilateral adrenalectomy (i.e. the CD tumor) from

8 cases. Within those 8 paired Cushing's/Nelson's tumors, 4 pairs were wild-type and 2 were mutant (2 deletions p.Ser718del), while the remaining two pairs consisted of wild-type CD tumors with *USP8* mutant NS tumors (p.Pro720Arg and p.Ser718del). Pathological investigation did not identify tumor part in these two CD specimens suggesting that what we analyzed was in fact non-tumor pituitary tissue.

We excluded three cases from subsequent statistical analyses. First, a female patient with c.2159C>A (p.Pro720Arg) mutation was also found to have the germline *AIP* variant c.911G>A (p.Arg304Gln) (22). This patient had a recurrent NS tumor and was operated twice. The *USP8* mutation c.2159C>A (p.Pro720Arg) was detected in the NS tumor specimens from both operations. Second, a male patient with wild-type *USP8* had a somatic mutation in *TP53* acquired after radiotherapy (12). Third, a female patient with wild-type *USP8* status had an invasive adenoma that progressed to a pituitary carcinoma with metastasis in the dura (3).

USP8 mutations were detected exclusively in female patients (14/27; 51.9%). No significant association was observed between *USP8* mutational status and most clinical and pathological parameters (Table 1). The time lapse between age at diagnosis of CD and NS tended to be longer in females with tumors positive for *USP8* mutation (median 13 vs 5 years; $P = 0.097$; $n = 26$; Supplementary Table 1, see section on supplementary data given at the end of this article). We did not observe differences in the distribution of patients treated with radiotherapy ($n = 24$); from the 13 patients with wild-type tumors, 9 were treated with radiotherapy (3 pre- and 6 postoperatively), and from the 12 patients with *USP8* mutant tumors, 7 were treated with radiotherapy (2 pre- and 5 postoperatively). Regarding the hormonal phenotype, the pairwise analysis showed that the postoperative levels of ACTH were higher in patients with *USP8* mutant tumors (639.8 vs 112.0 pg/mL; Mann–Whitney *U*, $P = 0.03$; $n = 23$; Table 1). We compared ACTH dynamics at the three given times (basal, preoperative and postoperative) in each group by means of Kruskal–Wallis and Friedman's tests corrected for multiple comparisons and we observed that the levels of plasma ACTH decreased significantly after NS surgery in the group of patients with *USP8* wild-type tumors (Mann–Whitney *U* for independent measurements, $P < 0.001$; Wilcoxon signed ranks for repeated measurements, $P = 0.001$; Fig. 2) but not in the *USP8* mutant group (Mann–Whitney *U*, $P = 0.999$; Wilcoxon signed ranks sums, $P = 0.283$). ACTH normalization was achieved only in few patients regardless of *USP8* status (3/13 vs 1/10, $P = 0.604$; $n = 23$).

Figure 1

Prevalence of *USP8* mutations in Nelson's syndrome (left) and Cushing's disease (right). Data on CD were obtained from references (14, 15, 16, 17).

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Table 1 Clinical features and *USP8* mutational status.

	Study cohort	Wild-type	Mutant	P value
Sex, n (%)				0.228
Females	27/30 (90.0)	13/27 (48.1)	14/27 (51.9)	
Males	3/30 (10.0)	3/3 (100.0)	0/3 (0.0)	
Age, years, median (IQR)				
At diagnosis of CD	30.0 (7.0)	31.0 (8.0)	30.0 (7.0)	0.403
At BADx	34.0 (13.0)	35.0 (17.0)	32.0 (10.0)	0.680
At diagnosis of NS	42.0 (16.0)	42.0 (21.0)	43.0 (14.0)	0.723
Time interval, years, median (IQR)				
From CD to NS	8.0 (13.8)	5.0 (14.0)	12.0 (13.0)	0.144
From CD to BADx	1.0 (3.8)	0.5 (4.0)	1.0 (3.3)	0.722
From BADx to NS	5.0 (8.0)	4.5 (7.3)	5.0 (10.0)	0.617
Body mass index, kg/m ² , mean (s.d.)	26.1 (5.3)	26.9 (5.4)	25.4 (5.4)	0.617
Hyperpigmentation, n (%)	25/27 (92.6)	12/14 (85.7)	13/13 (100.0)	0.481
Visual field defects, n (%)	6/27 (22.2)	4/14 (28.6)	2/13 (15.4)	0.648
Cranial nerve palsy, n (%)	6/26 (23.1)	4/13 (30.8)	2/13 (15.4)	0.645
Plasma ACTH, pg/mL, median (IQR)				
Basal ^a	100.0 (334.2)	104.7 (158.6)	100.0 (1353.0)	0.349
Preoperative ¹	1545.4 (6816.8)	1261.0 (2381.5)	1700.0 (11115.5)	0.072
Postoperative ¹	204.6 (620.5)	112.0 (251.8)	639.8 (1706.5)	0.030
ACTH normalization (<50 pg/mL), n (%)	4/23 (17.4)	3/13 (23.1)	1/10 (10.0)	0.604
Radiation therapy, n (%)				
Before sample collection	5/25 (20.0)	3/13 (23.1)	2/12 (16.7)	1.000
After sample collection	11/25 (44.0)	6/13 (46.2)	5/12 (41.7)	1.000
Number of sellar surgeries before BADx, n (%)				0.299
0	11/29 (37.9)	5/15 (33.3)	6/14 (42.9)	
1	11/29 (37.9)	8/15 (53.3)	3/14 (21.4)	
2	6/29 (20.7)	2/15 (13.3)	4/14 (28.6)	
>2	1/29 (3.5)	0/15 (0.0)	1/14 (7.1)	
Number of sellar surgeries after BADx, n (%)				0.683
1	21/27 (77.8)	9/13 (69.2)	12/14 (85.8)	
2	3/27 (11.1)	2/13 (15.4)	1/14 (7.1)	
>2	3/27 (11.1)	2/13 (15.4)	1/14 (7.1)	
Tumor size, mean (s.d.)	18.6 (9.6)	18.6 (10.5)	18.6 (9.3)	0.970
Tumor control, n (%)	18/24 (0.75)	9/13 (69.2)	9/11 (82.8)	0.410
Disease-related death, n (%)	4/27 (16.7)	3/15 (20.0)	1/12 (8.3)	0.605

BADx, bilateral adrenalectomy.

^aP values generated from Mann–Whitney *U* pairwise analysis. Bold value indicates statistical significance (*P*<0.05)

Discussion

In this study, we report for the first time the presence of somatic genetic defects in a large series of patients with NS tumors. In our cohort, almost half of the NS tumors contained somatic mutations in *USP8*, all of them causing substitution or deletion of the residues Ser718 or Pro720. This prevalence (45.5%) is similar to that observed in CD tumors (47.3%) in which the wide majority of the identified mutations affects the residues Ser718, Pro720 or both (14, 15, 16, 17). Similarly, the gender-specific distribution of mutations showed a female predominance for both CD and NS. In detail, half of the female patients presented tumors with *USP8* mutations, a frequency that resembles that observed for CD (14, 15, 16, 17). These similarities as well as the consistency in the *USP8* mutational status between

paired CD/NS samples strongly support the hypothesis of NS tumors as a progression or a recurrence of the initial CD tumor (2, 8). However, the same prevalence of *USP8* mutations between CD and NS tumors makes it unlikely that these mutations trigger the progression from Cushing's to Nelson's tumor.

Previous studies on CD have reported significant associations between *USP8* mutations and a younger age at diagnosis in female patients, smaller tumor size, higher basal levels of plasma ACTH and higher postoperative levels of urinary-free cortisol (13, 14, 15, 16, 17). We did not observe any association with these variables in our series of NS, probably due to the cohort size as well as the retrospective and multicentric nature of the study. Nevertheless, we did observe a significantly impaired reduction in plasma ACTH after NS surgery in patients with *USP8* mutated tumors. Postoperative hormone

Figure 2

Dynamics of plasma ACTH in patients with Nelson's syndrome with and without *USP8* mutations. Plasma ACTH was measured at the time of diagnosis with CD (basal), before (PreOP) and after the NS surgery (PostOP). Values are shown in a logarithmic scale. Lines represent the medians, boxes the 25th and 75th percentiles and whiskers the ranges of the distribution. * $P < 0.05$; ** $P < 0.001$; ns not significant. Statistical analysis revealed different ACTH dynamics within the wild-type group (basal vs PreOP, $P = 0.028$; PreOP vs PostOP, $P < 0.001$) and the *USP8* mutant group (basal vs PreOP, $P < 0.001$; PreOP vs PostOP, $P = 0.2565$) after correction for multiple comparisons. The differences between wild-type and mutants at basal ($P = 1.000$), PreOP ($P = 1.000$) and PostOP ACTH levels ($P = 0.630$) were not significant.

levels have been proposed as prognostic markers for surgical outcome, with several studies showing that low postoperative ACTH and cortisol levels are related to long-term remission and lower recurrence rates in CD (23, 24, 25, 26, 27). Therefore, the presence of *USP8* mutations may predict a worse postoperative phenotype.

Interestingly, in our cohort, we detected a somatic *USP8* mutation in a 27-year-old patient with an associated germline *AIP* variant c.911G>A (p.Arg304Gln). The patient showed early manifestation and rapid progression of the disease with the NS tumor being resected twice (22). The p.Arg304Gln variant has been seen as pathogenic from a clinical point of view, but according to the *in vitro* studies, it is considered a variant of unknown significance (28). This is the first reported case of a *USP8* mutant tumor associated with a germline *AIP* alteration. Since CD is rarely seen in patients with germline *AIP* mutation (29, 30, 31, 32, 33, 34, 35),

one may hypothesize that the acquisition of a somatic *USP8* mutation in this patient may have shifted toward the development of a corticotroph tumor.

This patient was treated with the second-generation multireceptor targeting somatostatin analog pasireotide and showed reduction in plasma ACTH and substantial improvement of symptoms after 6 months of treatment (22). This is in agreement with another study reporting good response to pasireotide treatment in patients with NS, thereby providing the rationale for their use in the management of corticotroph tumors (22, 36). The recent report of a direct relationship between *USP8* mutational status and somatostatin receptor 5 (SSTR5) expression (37) suggests that patients with *USP8* mutation positive corticotroph tumors may respond favorably to pasireotide treatment.

In conclusion, in this multicenter study we found that *USP8* mutations are common in NS tumors, showing a comparable prevalence to CD tumors. Similar to CD, there is a striking female predominance and an association with a worse postoperative outcome. Taken together, our data indicate that the *USP8* mutational status does not drive the corticotroph tumor progression that leads to NS. Hence, other yet-to-be-identified genetic alterations may determine the development of this aggressive tumor entity.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/EJE-17-0634>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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