The *USP8* mutational status may predict drug susceptibility in corticotroph adenomas of Cushing's disease

Kyohei Hayashi^{1,2}, Naoko Inoshita², Kohei Kawaguchi³, Arif Ibrahim Ardisasmita³, Hisanori Suzuki¹, Noriaki Fukuhara⁴, Mitsuo Okada⁴, Hiroshi Nishioka^{4,5}, Yasuhiro Takeuchi^{1,5}, Masayuki Komada³, Akira Takeshita^{1,5} and Shozo Yamada^{4,5}

Departments of ¹Endocrinology and Metabolism and ²Pathology, Toranomon Hospital, 2-2-2 Toranomon, Minato, Tokyo 105-8470, Japan, ³Department of Biological Sciences, Tokyo Institute of Technology, 4259-B16 Nagatsuta, Midori, Yokohama 226-8501, Japan, ⁴Department of Hypothalamic and Pituitary Surgery, Toranomon Hospital, 2-2-2 Toranomon, Minato, Tokyo 105-8470, Japan and ⁵Okinaka Memorial Institute for Medical Research, 2-2-2 Toranomon, Minato, Tokyo 105-8470, Japan

Correspondence should be addressed to A Takeshita
Email
coactivator@mac.com

Abstract

Context: Somatic mutations in the ubiquitin-specific peptidase *USP8* gene were recently detected in one- to two-third(s) of corticotroph adenomas of Cushing's disease (CD). These mutations may lead to the deubiquitination of EGFR, thereby increasing EGFR signaling, which has been implicated in ACTH hypersecretion.

Objective: Our objective was to determine the impact of *USP8* mutations on the clinicopathological features of CD.

Subjects and methods: *USP8* mutations as well as clinicopathological characteristics were examined in 60 corticotroph adenomas including 15 Crooke's cell adenomas (CCAs), a rare histological variant presenting with generally aggressive behavior, using qRT-PCR and/or immunohistochemistry.

Results: *USP8* mutations were exclusively detected in women, except for one case, with a prevalence of 42.2% in non-CCA and 13.3% in CCA (overall 35%). Clinically well-behaved presentations including microadenoma and curative resection were more common in mutated cases. The expression of EGFR was not associated with the mutation status. In contrast, mutated tumors expressed significantly higher levels of *POMC*, *SSTR5*, and *MGMT*.

Conclusions: Microadenomas that strongly express *POMC* were common among mutated tumors, which may lead to the mechanisms by which very small adenomas secrete excess ACTH to present overt CD. While *USP8* mutations were less likely to enhance tumorous ACTH hypersecretion via EGFR-mediated activation, the presence of *USP8* mutations may predict favorable responses to the somatostatin analog pasireotide, which exhibits high affinity for *SSTR5*. In contrast, non-mutated aggressive tumors such as CCA may respond better to the alkylating agent temozolomide because of their significantly weak expression of *MGMT*.

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Introduction

Cushing's disease (CD) is caused by an ACTH-secreting corticotroph adenoma that induces hypercortisolism. Most ACTH-secreting adenomas are microadenomas (<10 mm in maximum diameter), while macroadenomas (≥10 mm) are detected in 10–20% of patients. The first-line treatment for CD is transsphenoidal surgery (TSS).

The selective surgical removal of a microadenoma generally results in a cure with a low recurrence rate. In contrast, macroadenomas are typically more invasive tumors and often recur after surgery.

Crooke's cell adenoma (CCA), a histological variant type of corticotroph adenomas, is characterized by a

prominent hyalinized cytoplasm (Crooke's hyaline change) in most adenoma cells. Although CCA is a rare subtype of CD with an estimated prevalence of 4.4–14% among all corticotroph adenomas, it is generally aggressive, presenting as invasive macroadenomas, is resistant to surgery and radiotherapy, and has a high recurrence rate. The development of corticotroph carcinomas in preexisting CCA is not uncommon (1, 2).

The four members of the ErbB family, which includes epidermal growth factor receptor (EGFR) and ERBB2-4, are cell surface receptor tyrosine kinases (TKs) (3). When tumor cells overexpress ErbB receptors, they exhibit aggressive tumor cell growth. Previous studies reported that pituitary adenomas including corticotroph adenomas had variable EGFR expression (4, 5), which correlated with tumor aggressiveness (6, 7, 8). Somatic mutations in the 14-3-3 protein binding motif of the deubiquitinating enzyme (DUB) gene, ubiquitin specific peptidase 8 (*USP8*), were recently detected in approximately one- to two-third(s) of corticotroph adenomas (9, 10). These mutations potentially lead to the deubiquitination of EGFR, thereby increasing EGFR signaling, which has been implicated in corticotroph tumorigenesis. Furthermore, *in vitro* studies revealed that *USP8* mutations enhanced the promoter activity of the gene encoding proopiomelanocortin (*POMC*) through stabilized EGFR signaling, which may explain ACTH hypersecretion in patients with CD (9, 10, 11). These findings have raised the question as to whether *USP8* mutations are associated with the clinical features of CD such as tumor aggressiveness, ACTH hypersecretion, surgical remission rate, and drug susceptibility to the dopamine agonist cabergoline and somatostatin analog pasireotide (12, 13), as well as the alkylating agent temozolomide, which is used in the treatment of aggressive pituitary adenomas and carcinomas (2, 14, 15, 16).

Clinical presentation such as the tumor size, extent of cavernous sinus (CS) invasion, surgical outcome, and Ki-67 labeling index has been associated with tumor aggressiveness (15, 16). The positive expression of dopamine receptor D2 (DRD2), somatostatin receptors (SSTRs), and EGFR have been shown to predict drug susceptibility to dopamine agonists, somatostatin analogs, and TK inhibitors respectively (12, 13, 17). In contrast, the negative expression of O⁶-methylguanine DNA methyltransferase (MGMT) and the DNA mismatch repair protein MSH6 have been associated with tumor responsiveness (18, 19) and resistance to temozolomide (20) respectively. In order to determine the impact of *USP8* mutations in CD, we herein evaluated the clinicopathological features of 60

corticotroph adenomas including 15 CCAs using quantitative real-time PCR (qRT-PCR) and/or immunohistochemistry, and evaluated their relationships with the *USP8* mutation status.

Subjects and methods

Patients

We reviewed all tumor samples from 209 patients with clinically active CD who underwent TSS between March 2004 and October 2013. In all cases, surgery was performed by the same surgeon (SY) who has experience with more than 2500 pituitary surgeries. Samples were examined by routine histological methods and immunohistochemical staining as previously described (21, 22). They were classified as CCA when the percentage of Crooke's hyaline change in tumor cells was >50%, with the remaining tumor samples being considered as non-Crooke's cell adenoma (non-CCA) (1, 21). Sixteen tumor samples were identified as CCA from 209 samples (7.7%), and 15 out of 16 samples were available for RNA extraction. They were compared with 45 tumor samples selected from recent non-CCA tumor samples. The control group comprised three normal anterior pituitary tissues obtained from patients during surgery for craniopharyngioma without hypopituitarism preoperatively. Informed consent was obtained from all patients and tissue collection was approved by the Institutional Review Board of Toranomon Hospital. Preoperative contrast-enhanced MRI (1.5 or 3.0 T) was performed in all patients. CS invasion was confirmed by both operative and histological findings. Patients were considered to be in surgical remission on the basis of postoperative serum cortisol levels <3 µg/dl (23).

RNA extraction and cDNA preparation

Pituitary tumor samples were stored in RNAlater (Life Technologies) at –80 °C until RNA extraction. Total RNA was prepared using RNeasy mini (Qiagen). PrimeScript RT reagent (Takara Bio, Otsu, Japan) was used for the preparation of cDNA.

qRT-PCR

With the resulting cDNA as a template, qRT-PCR was performed with Power SYBR Green PCR Master Mix (Life Technologies) and gene-specific primers (see [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article) in a StepOnePlus real-time PCR

system (Life Technologies). Relative quantification was performed using standard curve methods. mRNA expression results were normalized to *GAPDH* and expressed in arbitrary units (AU).

Direct sequencing for detection of *USP8* mutations

Tumor cDNA was amplified by PCR using the forward primer, 5'-GGGAGGATCGTACCAGGACT-3' in exon 13 and the reverse primer 5'-AGGTTCCGAATCTGA-GAAGCA-3' in exon 14, corresponding to codon 645–764 of *USP8* (GenBank accession number: NM_005154.4). Direct sequencing was performed using internal primers.

Biochemistry

All experimental procedures regarding *in vitro* experiments on COS-7 and HeLa cells, including the construction of cDNA expression plasmids, cell culture, DNA transfection, immunoprecipitation and immunoblotting, DUB assays, and immunofluorescence staining were performed as previously described (9).

Immunohistochemistry

A summary of the primary antibodies used and working dilutions are provided in [Supplementary Table 2](#), see section on [supplementary data](#) given at the end of this article. Slides were stained using an EnVision+System-HRP Kit (Dako, Glostrup, Denmark). Immunohistochemical reactions were developed with diaminobenzidine and slides were counterstained with hematoxylin.

EGFR, SSTR2, SSTR5, DRD2, MGMT, and MSH6 protein expression levels were scored using the immunoreactive score (IRS) as previously reported (24, 25); IRS (0–12) was the product of the proportion of immunoreactive cells (0, 0%; 1, <10%; 2, 10–50%; 3, 51–80%; or 4, >80%) multiplied by the staining intensity (0, no staining; 1, weak; 2, moderate; and 3, strong). The scores of the images are shown in [Fig. 1](#).

Statistical analysis

Data were expressed as a median with an interquartile range (IQR). Comparisons between categorical variables were performed using Fisher's exact test. Comparisons between numerical variables were performed using the Mann–Whitney *U* test. Correlations were determined by calculating Spearman's rank correlation coefficient. *P* values of <0.05 were considered significant. A stepwise

forward-selection multivariate regression analysis was employed with either surgical remission, mRNA expression, or the IRS score as a dependent variable and different parameters including the *USP8* mutation status, *USP8* mRNA expression, pathological finding (non-CCA or CCA), sex, plasma ACTH concentration, maximum tumor diameter, Knosp classification, cavernous sinus invasion, and Ki-67 labeling index as independent variables (*n*=51, after excluding nine subjects whose tumorous RNA samples may have been contaminated with a significant amount of the normal anterior pituitary).

Results

Clinical characteristics of subjects

A previous *in vitro* study suggested that *USP8* mutations were associated with EGFR activation of corticotroph adenomas. Therefore, we speculated that the aggressive type of CD, CCA may be more likely to demonstrate *USP8* mutations such that EGFR protein expression levels may be higher in CCA than in non-CCA. Therefore, we evaluated 60 samples comprising 15 CCA and 45 non-CCA. The ratio of CCA (25%) in the present study was markedly higher than the actual prevalence of CCA (7.7%) in our hospital.

The clinical characteristics of the tumors examined are summarized in [Table 1](#). Note that specimens of very small microadenomas, such as <5 mm, were generally used for diagnostic purposes only, and were rarely available for the present study, lending a certain selection bias to the non-CCA group. CCA were significantly larger than non-CCA (maximum tumor diameter, median 19.2 mm vs 9.4 mm, *P*=0.002) ([Supplementary Figure 1A](#), see section on [supplementary data](#) given at the end of this article). Macroadenomas were more frequent among CCA than among non-CCA (93.3% vs 40%, *P*<0.001). The prevalence of CS invasion was significantly higher in CCA than in non-CCA (80% vs 48.9%, *P*=0.041). The surgical remission rate was lower in CCA than in non-CCA although there was no statistic difference (46.7% vs 75.6%, *P*=0.055). Clinical features including age at diagnosis, sex ratio, plasma ACTH, serum cortisol, the Ki-67 labeling index, and prior history of TSS were not significantly different between the two groups. Taken together, our CCA cases confirmed the aggressive clinical features of invasive macroadenomas with a low surgical remission rate.

Score	0 (negative, 0 – 1)	2+ (low, 2 – 3)	6+ (medium, 4 – 8)	(12+)
EGFR
Score	0 (negative, 0 – 1)	2+ (low, 2 – 3)	4+ (medium, 4 – 8)	9+ (high, 9 – 12)
SSTR2
Score	1+ (negative, 0 – 1)	2+ (low, 2 – 3)	6+ (medium, 4 – 8)	12+ (high, 9 – 12)
SSTR5
Score	0 (negative, 0 – 1)	2+ (low, 2 – 3)	8+ (medium, 4 – 8)	(12+)
DRD2
Score	0 (negative, 0 – 1)	3+ (low, 2 – 3)	8+ (medium, 4 – 8)	12+ (high, 9 – 12)
MGMT
Score	0 (negative, 0 – 1)	2+ (low, 2 – 3)		12+ (high, 9 – 12)
MSH6

Figure 1

Examples of immunoreactive scores (IRS, 0–12) for EGFR, SSTR2, SSTR5, DRD2, MGMT, and MSH6. Sections were counterstained with hematoxylin. The highest IHC scores for EGFR and DRD2

were 6+ and 8+ respectively. Positive controls (colon cancer for EGFR and prolactinoma for DRD2) were included (original magnification, $\times 400$).

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Table 1 Comparisons of clinical characteristics of non-CCA vs CCA and WT vs *USP8*-mutated adenoma.

	Total	Non-CCA	CCA	P	WT	<i>USP8</i> mutant	P
Patients	60	45 (75%)	15 (25%)		39	21	
Non-CCA					26 (66.7%)	19 (90.5%)	0.061 ^a
CCA					13 (33.3%)	2 (9.5%)	
Age at diagnosis (years)							
Median	45	41	45	0.22 ^b	44	37	0.42 ^b
Range	19–81	19–65	22–81		22–81	19–65	
IQR	36.3–57	28.5–51.5	37–59		34–55	28.5–51	
Sex							
Males	13 (21.7%)	9 (20%)	4 (26.7%)	0.72 ^a	12 (30.8%)	1 (%)	0.023 ^a
Females	47 (78.3%)	36 (80%)	11 (73.3%)		27 (69.2%)	20 (%)	
Plasma ACTH (pg/ml)							
Median	102.75	97.6	111	0.19 ^b	110.1	79.1	0.036 ^b
Range	27.3–1950	27.3–287.0	32.2–1950.0		27.3–1950	28.6–220.2	
IQR	70.1–141.8	67.55–132.8	78.1–228.0		78.1–221.0	57.0–112.5	
Serum cortisol (μg/dl)							
Median	17.4	19.7	21.4	0.52 ^b	20.9	19.3	0.97 ^b
Range	9.7–54.0	9.7–54.0	12.5–44.6		9.7–52.4	11.7–54.0	
IQR	15.6–23.8	15.7–27.6	17–30.2		16.3–29.4	15.65–27.6	
Maximum tumor diameter (mm)							
Median	10.7	9.4	19.2	0.0023 ^b	16.3	9.1	0.0012 ^b
Range	3.5–45.6	3.5–45.6	4.9–40.3		4.0–45.6	3.5–18.6	
IQR	7.4–21.0	6.9–17.2	14.1–25.5		8.4–25.5	6.0–12.3	
Microadenoma	28 (46.7%)	27 (60%)	1 (6.7%)	0.0003 ^a	14 (35.9%)	14 (66.7%)	0.031 ^a
Macroadenoma	32 (53.3%)	18 (40%)	14 (93.3%)		25 (64.1%)	7 (33.3%)	
Knosp classification							
0	24 (40%)	21 (46.7%)	3 (20%)	0.053 ^b	12 (30.8%)	12 (57.1%)	0.012 ^b
1	12 (20%)	8 (17.8%)	4 (26.7%)		8 (20.5%)	4 (19.0%)	
2	10 (16.7%)	8 (17.8%)	2 (13.3%)		6 (15.4%)	4 (19.0%)	
3	1 (1.7%)	1 (2.2%)	0 (0%)		0 (0%)	1 (4.8%)	
4	13 (21.7%)	7 (15.6%)	6 (40%)		13 (33.3%)	0 (0%)	
Median	1	1	2		1	0	
Range	0–4	0–4	0–4		0–4	0–3	
IQR	0–2	0–2	1–4		0–4	0–1	
Cavernous sinus invasion							
Negative	26 (43.3%)	23 (51.1%)	3 (20%)	0.041 ^a	14 (35.9%)	12 (57.1%)	0.17 ^a
Positive	34 (56.7%)	22 (48.9%)	12 (80%)		25 (64.1%)	9 (42.9%)	
Ki-67 index							
≤3%	37 (61.7%)	26 (57.8%)	11 (73.3%)	0.37 ^a	24 (61.5%)	13 (61.9%)	1.0 ^a
>3%	23 (38.3%)	19 (42.2%)	4 (26.7%)		15 (38.5%)	8 (38.1%)	
Median	2.7	2.9	2.5	0.89 ^b	3	2.5	0.83 ^b
Range	0.1–30.0	0.1–30.0	0.5–20.0		0.1–30.0	0.1–12.0	
IQR	1.0–5.0	1.0–5.0	1.0–5.0		1.0–5.0	1.0–5.0	
1st TSS	38 (63.3%)	29 (64.4%)	9 (60%)	0.77 ^a	21 (53.8%)	17 (81.0%)	0.051 ^a
Prior history of TSS	22 (36.7%)	16 (35.6%)	6 (40%)		18 (46.2%)	4 (19.0%)	
Surgical remission	41 (68.3%)	34 (75.6%)	7 (46.7%)	0.055 ^a	21 (53.8%)	20 (95.2%)	0.001 ^a
Persistent	19 (31.7%)	11 (24.4%)	8 (53.3%)		18 (46.2%)	1 (4.8%)	
Metastasis		0	1		1	0	

^aFisher's exact test.^bMann-Whitney *U*-test.

Determination and relative mRNA expression of *USP8* mutants

The PCR-amplified cDNA fragments for codon 645–764 of *USP8* containing the 14-3-3 binding motif were sequenced. *USP8* mutations were detected in 21 (35%) out of 60 adenomas. Sanger sequencing showed WT and

mutant alleles in the mutated adenomas, consistent with heterozygous mutations. These mutations were S718del (*n*=9), P720R (*n*=6), S719P (*n*=2), S718F (*n*=1), S718_P720delinsT (*n*=1), P720Q (*n*=1), and P720_D721del (*n*=1) (Fig. 2A). Representative sequences of the mutations are shown in Supplementary Figure 2, see

section on [supplementary data](#) given at the end of this article. S718_P720delinsT was a novel mutation. Others (S718del, P720R, S719P, S718F, P720Q, and P720_D721del) have been previously identified (9, 10, 11).

USP8 mRNA levels were measured using qRT-PCR to determine whether the expression levels of *USP8* differed between the *USP8* mutants and the WT. In order to exclude the possibility of normal pituitary contamination in the RNA samples of 60 extracted corticotroph adenoma tissues, qRT-PCR was performed using primers specific for *POU1F1* (*PIT1*), which is expressed in thyro-mammotroph-derived cells, but not in corticotroph lineages. Fifty-one samples (21 out of 26 WT in non-CCA, 15 out of 19 *USP8* mutants in non-CCA, 13 out of 13 WT in CCA, and two out of two *USP8* mutants in CCA) expressed <2% *POU1F1* mRNA relative to the mean expression of three normal anterior pituitary samples, and were used in subsequent qRT-PCR analyses. However, the remaining nine RNA samples were excluded from subsequent qRT-PCR analyses because they expressed more than 8% *POU1F1* mRNA relative to the mean normal pituitary samples (Supplementary Figure 1B, see section on [supplementary data](#) given at the end of this article). Eight out of the nine excluded samples were microadenomas. *USP8* mRNA expression was significantly higher in the mutants than in the WT *USP8* (median 4.99 (AU) vs 2.59 (AU), $P=0.021$) (Supplementary Figure 1C).

Functional analysis of *USP8* mutations

Previously-reported *USP8* mutants loose binding to 14-3-3 proteins, undergo cleavage adjacent to the 14-3-3 binding

motif to the 90- and 40-kDa fragments, and the 40-kDa fragment (C40) acquires high DUB activity (9, 11). Since the functions of three of the seven *USP8* mutants identified in this study (S718F, S719P, and S718_P720delinsT) had not been determined, we performed a functional analysis. Co-immunoprecipitation experiments showed that 14-3-3 binding of C-terminally Flag-tagged S718F, S719P, and S718_P720delinsT was markedly affected, similar to the previously-characterized mutant P720R in mammalian COS-7 cells (Fig. 2B).

When the C-terminally Flag-tagged mutants were immunoprecipitated from transfected COS-7 cells and eluted from the antibody with the Flag peptide, cleaved C40 was detected in addition to the full-length 130-kDa form in the three mutants but hardly in WT *USP8* (Fig. 2C). C40 levels were consistently higher in S718F than in the other two mutants (Fig. 2D, left).

In order to determine DUB activity, Flag-tagged *USP8* proteins were immunopurified from transfected COS-7 cells, as in Fig. 2C, and their purity, concentration, and partial cleavage were assessed by Coomassie staining (Fig. 2D, left). Approximately the same amounts of enzymes were incubated with Lys63-linked ubiquitin chains. Ubiquitin chains were more efficiently hydrolyzed by all *USP8* mutants than by WT *USP8* (Fig. 2D, right). Consistent with S718F being more efficiently cleaved than other mutants (Fig. 2D, left), it exhibited the highest DUB activity (Fig. 2D, right).

The effects of *USP8* mutations on the downregulation of EGFR were examined in HeLa cells treated with 100 ng/ml EGF for 1 h. While the overexpression of WT *USP8* did not affect the endocytosis of ligand-activated

Figure 2

(A) Summarized diagram of somatic *USP8* mutations in 21 out of 60 corticotroph adenomas. All mutations were detected between amino acids 718 and 721 in the hotspot region overlapping the 14-3-3 binding motif. (B) 14-3-3 protein binding of *USP8* mutants. Top and middle) Anti-14-3-3 protein (top) and anti-Flag (middle) immunoblots of the anti-Flag immunoprecipitates of lysates of COS-7 cells expressing Flag-tagged *USP8* proteins. (Bottom) Anti-14-3-3 immunoblots of the lysates of COS-7 cells expressing Flag-tagged *USP8* proteins. (C) Susceptibility of *USP8* mutants to cleavage. Anti-Flag immunoblots of Flag-tagged *USP8* proteins expressed in COS-7 cells, immunoprecipitated from their lysates with an anti-Flag antibody, and eluted from the antibody with the Flag peptide. Open and closed arrowheads indicate the full-length and

cleaved *USP8* proteins respectively. (D) DUB activity of *USP8* mutants. (Left) Coomassie staining of Flag-tagged *USP8* proteins expressed in COS-7 cells and immunopurified from their lysates, as in (C). (Right) Anti-ubiquitin immunoblots of K63-linked ubiquitin chains (dimer-heptamer, Ub2-7) incubated with the immunopurified Flag-tagged *USP8* proteins shown on the left. Arrowheads indicate each ubiquitin chain. (E) Effects of *USP8* mutants on EGFR downregulation. Immunofluorescence staining of HeLa cells transfected with the Flag-tagged *USP8* proteins and treated with 100 ng/ml EGF for 1 h. Cells were triple-stained with an anti-Flag antibody (magenta, top), anti-EGFR antibody (green, bottom), and DAPI (blue, bottom). Asterisks indicate cells not expressing Flag-*USP8* proteins. Scale bars, 10 μ m.

EGFR, that of the three new mutants led to the retention of EGFR on the plasma membrane, suggesting that elevated DUB activity in the mutants inhibited the downregulation of EGFR (Fig. 2E).

Clinical characteristics of the *USP8* mutant group

The clinical features of the two groups, the WT and mutants, are summarized on the right side of Table 1, while those of four groups, the WT and mutants in non-CCA or CCA, are summarized in Supplementary Table 3, see section on supplementary data given at the end of this article. Mutations were more frequent in non-CCA (19 out of 45, 42.2%) than in CCA (two out of 15, 13.3%), and observed exclusively in females (20 out of 47), and rarely in males (one out of 13). Plasma ACTH levels were significantly lower in the mutant than in the WT group, whereas no significant difference was observed for serum cortisol. Tumors with *USP8* mutations were significantly smaller than WT tumors, and all mutants were <2 cm in size (Supplementary Figure 1A). Surgical remission was achieved in 20 out of 21 patients (95.2%) in the mutant group, but in only 21 out of 39 (53.8%) in the WT group (16 out of 26 (61.5%) WT in non-CCA and five out of 13 (38.5%) WT in CCA) (Supplementary Table 3). The stepwise forward-selection multivariate regression identified the maximum tumor diameter and *USP8* mutation status as contributors to surgical remission (adjusted $R^2=0.493$, $P<0.0001$). No significant differences were observed in clinical features including age at diagnosis, the prevalence of CS invasion, and the Ki-67 labeling index between the WT and mutant groups. Taken together, *USP8* mutations were typically detected in women and characterized as small non-aggressive tumors with a high surgical remission rate.

Relative mRNA and protein expression of EGFR

We determined the mRNA and protein expression levels of EGFR using qRT-PCR and Immunohistochemistry (IHC) respectively. No significant differences were observed in *EGFR* mRNA or IHC expression between WT and mutants (Fig. 3A and B), indicating that *USP8* mutations did not affect the total protein level of EGFR. However, EGFR protein expression was significantly higher in CCA than in non-CCA (median IRS 1+ vs 0, $P=0.023$) (Fig. 3B), suggesting that the generally aggressive behavior of CCA was partly attributed to dominant EGFR protein expression. The stepwise regression analysis identified pathological findings (non-CCA or CCA) as the only

Figure 3

Expression of EGFR in corticotroph adenomas. (A) Comparison of *EGFR* mRNA expression levels between WT and *USP8* mutant corticotroph adenomas in non-CCA or CCA. Expression levels of *EGFR* were analyzed by qRT-PCR, normalized to *GAPDH*, and expressed in arbitrary units (AU). (B) Comparison of EGFR IHC scores between WT and *USP8* mutant corticotroph adenomas in non-CCA or CCA. EGFR staining intensity was scored using immunoreactive scores (IRS, 0–12). Results are presented as box plots, and the line denotes the median values. The whiskers are 5th and 95th percentiles. Significance was determined by the Mann–Whitney *U* test.

contributor to the IHC score of EGFR (adjusted $R^2=0.131$, $P=0.0052$).

The subcellular EGFR immunoreactivity patterns of the nine EGFR-positive corticotroph adenomas ($\text{IRS} \geq 2+$) are shown in Supplementary Figure 3, see section on supplementary data given at the end of this article. It is important to note that Crooke's hyaline change, characterized by the massive accumulation of cytokeratin filaments in the cytoplasm, may affect the subcellular localization of EGFR. Although one case of WT in CCA showed the cytoplasmic as well as membrane expression of EGFR, the other eight cases showed similar membrane expression patterns of EGFR, suggesting no apparent effect of *USP8* mutations on the subcellular localization of EGFR.

Relative mRNA expression of POMC

In Fig. 4A, CCA showed significantly lower *POMC* mRNA levels than non-CCA (median 0.54 vs 1.84, $P=0.012$). *POMC* mRNA expression levels were significantly higher in *USP8* mutants than in WT *USP8* (median 2.49 vs 0.52, $P<0.001$). In Fig. 4B, a positive correlation was observed between *USP8* mRNA expression and *POMC* mRNA expression levels in the mutants ($r=0.60$, $P=0.017$), but

Figure 4

Expression of *POMC* mRNA in corticotroph adenomas. (A) Comparison of *POMC* mRNA expression levels between WT and *USP8* mutant corticotroph adenomas in non-CCA or CCA. Expression levels of *POMC* mRNA were analyzed by qRT-PCR, normalized to *GAPDH*, and expressed in arbitrary units (AU). (B) Relationship between relative *USP8* mRNA and relative *POMC* mRNA levels (WT, open circles; mutant, filled circles). (C) Relationship between maximum tumor diameter and relative *POMC* mRNA levels. Results are presented as box plots, and the line denotes the median values. The whiskers are 5th and 95th percentiles. Significance was determined by the Mann–Whitney *U* test (A). Spearman's rank correlation coefficient was used to calculate *R* and *P* values (B and C).

not in the WT ($r=0.23$, $P=0.19$), indicating *USP8* mutant-dependent *POMC* mRNA expression. Maximum tumor diameters and *POMC* mRNA expression levels showed a negative correlation ($r=-0.54$, $P<0.001$) (Fig. 4C). The stepwise regression analysis identified maximum tumor diameter and *USP8* mRNA expression as contributors to *POMC* mRNA expression (adjusted $R^2=0.256$, $P=0.0003$). Taken together, small tumors such as *USP8* mutants generally had higher *POMC* mRNA expression levels, whereas large tumors such as CCA generally had lower *POMC* mRNA expression levels.

Relative mRNA and protein expression of somatostatin and dopamine receptors

The mRNA and protein expression levels of *SSTR2* were both generally low in the entire cohort. No significant differences were observed in *SSTR2* mRNA or protein expression levels between WT and mutants or between non-CCA and CCA (Supplementary Figure 5A and B,

Figure 5

Expression of *SSTR5* in corticotroph adenomas. (A) Comparison of *SSTR5* mRNA expression levels between WT and *USP8* mutant corticotroph adenomas in non-CCA or CCA. Expression levels of *SSTR5* were analyzed by qRT-PCR, normalized to *GAPDH*, and expressed in arbitrary units (AU). (B) Comparison of *SSTR5* IHC scores between WT and *USP8* mutant corticotroph adenomas in non-CCA or CCA. *SSTR5* staining intensity was scored using immunoreactive scores (IRS, 0–12). (C) Relationship between relative *USP8* mRNA and relative *SSTR5* mRNA levels (WT, open circles; mutant, filled circles). (D) Relationship between relative *SSTR5* mRNA levels and *SSTR5* IHC scores (WT, open circles; mutant, filled circles). Results are presented as box plots, and the line denotes the median values. The whiskers are 5th and 95th percentiles. Significance was determined by the Mann–Whitney *U* test (A and B). Spearman's rank correlation coefficient was used to calculate *R* and *P* values (C).

see section on [supplementary data](#) given at the end of this article).

The mRNA expression of *SSTR5* was significantly higher in *USP8* mutants than in WT *USP8* (median 2.95 vs 0.06, $P<0.001$) (Fig. 5A). *USP8* mRNA and *SSTR5* mRNA expression levels showed a positive correlation (WT: $r=0.48$, $P=0.006$; mutants: $r=0.84$, $P<0.001$) (Fig. 5C). The stepwise regression analysis identified the *USP8* mutation status and *USP8* mRNA expression as contributors to *SSTR5* mRNA expression (adjusted $R^2=0.561$, $P<0.0001$). Similar to mRNA expression, *SSTR5* protein expression levels was significantly higher in the mutants than in the WT (median IRS 12+ vs 1+, $P<0.001$) (Fig. 5B). The IHC score was significantly lower in CCA than in non-CCA (median IRS 1+ vs 4+, $P=0.010$) (Fig. 5B). Cases of lower IRS were mainly found in the low *SSTR5* mRNA expression group of WT (Fig. 5D). The stepwise regression analysis identified the *USP8* mutation status and *USP8* mRNA expression as contributors to the IHC score of *SSTR5* (adjusted $R^2=0.425$, $P<0.0001$). The subcellular *SSTR5* immunoreactivity patterns of the 15 corticotroph adenomas that exhibited IRS scores of 12+ are shown in [Supplementary Figure 4](#), see section on [supplementary data](#) given at the end of this article. Crooke's hyaline change may have affected the subcellular localization of *SSTR5*. The WT and mutants showed similar membrane expression patterns of *SSTR5*.

In *DRD2*, mRNA and protein expression levels were both generally low in the entire cohort. However, *DRD2* mRNA expression levels were significantly higher in the *USP8* mutants of CCA than in the WT of CCA (median 0.81 vs 0.08, $P=0.038$) ([Supplementary Figure 5C](#), see section on [supplementary data](#) given at the end of this article). No significant difference was observed in protein expression levels between the WT and mutants or between non-CCA and CCA ([Supplementary Figure 5D](#)).

Relative mRNA and protein expression of MGMT

MGMT mRNA expression levels were significantly higher in *USP8* mutants than in the WT (median 3.47 vs 0.78, $P=0.001$) (Fig. 6A). In the IHC analysis, *MGMT* protein expression levels were significantly higher in the mutants than in the WT (median 8+ vs 1+, $P<0.0001$), and most *USP8* mutants (20 out of 21) were immunopositive for *MGMT* (IHC score ≥ 2) (Fig. 6B). *MGMT* mRNA and protein expression levels were significantly lower in CCA than in non-CCA, and most CCA (13 out of 15) were immunonegative for *MGMT* (Fig. 6A and B). *USP8* mRNA expression and *MGMT* mRNA expression levels showed

Figure 6

Expression of MGMT in corticotroph adenomas. (A) Comparison of *MGMT* mRNA expression levels between WT and *USP8* mutant corticotroph adenomas in non-CCA or CCA. Expression levels of *MGMT* were analyzed by qRT-PCR, normalized to *GAPDH*, and expressed in arbitrary units (AU). (B) Comparison of *MGMT* IHC scores between WT and *USP8* mutant corticotroph adenomas in non-CCA or CCA. *MGMT* staining intensity was scored using immunoreactive scores (IRS, 0–12). (C) Relationship between relative *USP8* mRNA and relative *MGMT* mRNA levels (WT, open circles; mutant, filled circles). (D) Relationship between relative *MGMT* mRNA levels and *MGMT* IHC scores (WT, open circles; mutant, filled circles). Results are presented as box plots, and the line denotes the median values. The whiskers are 5th and 95th percentiles. Significance was determined by the Mann–Whitney U test (A and B). Spearman's rank correlation coefficient was used to calculate R and P values (C).

a positive correlation (WT: $r=0.55$, $P=0.002$; mutants: $r=0.69$, $P=0.006$) (Fig. 6C). *MGMT* immunonegative tumors were mainly detected in the lower *MGMT* mRNA expression group of the WT (Fig. 6D). The stepwise regression analysis identified *USP8* mRNA expression levels and pathological findings (non-CCA or CCA) as contributors to *MGMT* mRNA expression (adjusted $R^2=0.579$, $P<0.0001$), and *USP8* mRNA expression levels,

the maximum tumor diameter, and pathological findings (non-CCA or CCA) as contributors to the IHC score of MGMT (adjusted $R^2=0.454$, $P<0.0001$).

Relative mRNA and protein expression of MSH6

MSH6 mRNA expression levels were significantly higher in *USP8* mutants and non-CCA than in the WT and CCA respectively (Supplementary Figure 5E, see section on supplementary data given at the end of this article). However, in the IHC analysis, most cases in the entire cohort (57 out of 60) showed high protein expression levels (12+), and there was no significant difference in protein expression levels between the WT and mutants or between non-CCA and CCA (Supplementary Figure 5F).

Discussion

We herein detected *USP8* mutations in 21 out of 60 (35%) Japanese patients with CD. Similar to previously reported mutants, new mutants (S719P, S718F, and S718_P720delinsT) lost 14-3-3 protein binding, underwent cleavage and catalytic activation, and suppressed EGFR downregulation. Among the three mutants examined, S718F was more prone to cleavage and exhibited higher DUB activity than the others, which is consistent with the critical role of Ser718 phosphorylation in *USP8* binding to 14-3-3 proteins (26) and high frequency of mutations at Ser718 in corticotroph adenomas (9, 10, 11).

The phenotype of typical *USP8* mutants represents a CD with small adenomas in middle-aged women. It may have caused the higher surgical remission rate of the mutants (95.2%) than that of the WT (53.8%) in our subjects. Similar to our results, a Chinese group showed that mutated adenomas were significantly smaller than WT adenomas (10), whereas a German group reported no significant differences (11). The reason(s) for the discrepancy are unclear but may be partly due to differences in sample recruitment. Specifically, samples were collected from one institution in our study and the Chinese study, but from seven institutions in the German study. The ethnic difference of the subjects may also have been a factor.

Using the three new *USP8* mutants, we confirmed *in vitro* that these mutations elevated DUB activity, thereby increasing the cell surface expression of EGFR (9, 10). However, there was no significant difference in EGFR protein expression levels between the WT and mutants in our IHC analysis. The monoclonal EGFR antibody, clone 31G7 used in our study is a validated antibody widely used

in IHC analyses of various cancers (27, 28). In our hospital, we routinely use this antibody for IHC of cancers such as colorectal cancer and breast cancer, suggesting that false negative errors were unlikely. The *in vitro* and *in vivo* discrepancies may have partly been due to the use of a supra-physiological concentration (100 ng/ml) of EGF in the *in vitro* experiments, which activated all EGFR molecules simultaneously. As the *USP8* mutants selectively inhibit the downregulation of ligand-activated, ubiquitinated EGFR, but do not affect inactive EGFR, the total EGFR protein level may not reflect the difference in the downregulation rate of activated EGFR *in vivo*. Therefore, further studies are warranted. In contrast, EGFR protein expression levels were significantly higher in CCA than in non-CCA in our IHC analysis. Thus, the aggressive phenotype of CCA may be partly explained by EGFR-mediated activation. In this regard, molecular targeted therapies such as TK inhibitors may be an alternate therapeutic option for refractory cases of CCA (17).

USP8 mRNA expression levels were higher in *USP8*-mutant than in *USP8*-WT corticotroph adenomas (Supplementary Figure 1C, see section on supplementary data given at the end of this article). Consistent with this result, *USP8* protein expression levels were shown to be higher in *USP8*-mutant than in WT corticotroph adenomas (9). A previous study reported that a dexamethasone treatment increased *USP8* protein expression levels in a glucocorticoid receptor-dependent manner in human embryonic kidney cells, suggesting the regulation of corticotroph *USP8* expression by the positive feedback system of the hypothalamic–pituitary–adrenal gland axis (29). However, this does not explain why *USP8*-mutant adenomas expressed *USP8* mRNA more strongly than WT adenomas because no significant differences were observed in serum cortisol levels between mutant and WT adenomas (Table 1). Therefore, the molecular mechanisms underlying the upregulation of *USP8* gene expression by its mutations in the protein-coding region (i.e. 14-3-3 binding motif) remain unknown.

We showed that mRNA and protein expression levels of *SSTR5* were significantly higher in the *USP8* mutant group than in the WT group. Information regarding the mechanisms of transcriptional regulation for the *SSTR5* gene is currently limited. It will be interesting to determine *in vitro* whether *USP8* mutants directly regulate *SSTR5* mRNA expression. However, the elevated expression of *SSTR5* mRNA in *USP8*-mutant adenomas may be an epiphenomenon to the tumor phenotype of the mutants. The higher *SSTR5* mRNA levels of the mutants may not solely explain the higher *SSTR5* protein

expression levels. SSTRs belong to the superfamily of G protein-coupled receptors (GPCRs). Similar to other GPCRs, agonist-stimulated SSTRs undergo desensitization and endocytosis (30). Recent studies revealed that the desensitization of GPCRs is mediated not only by phosphorylation and β -arrestins, but also by ubiquitination (31). Of particular interest is whether ubiquitinated SSTR5 is a substrate of USP8 because the elevated DUB activity of the *USP8* mutants may inhibit the down-regulation of SSTR5.

The somatostatin analog pasireotide, which possesses high SSTR5 affinity, has been reported to normalize urinary cortisol levels in ~25–30% of patients with CD (12, 32, 33, 34). The higher SSTR5 expression levels in *USP8* mutant adenomas suggest that the presence of *USP8* mutations may predict favorable responses to pasireotide in addition to their high surgical remission rate shown in the present study. In contrast, surgically refractory cases of *USP8* WT adenomas including CCA, which often present with invasive macroadenomas, may generally be resistant to medical therapy with pasireotide.

MGMT mRNA and protein expression levels were both significantly higher in *USP8* mutants than in the WT. Furthermore, compared with the WT, strong immunopositive expression of *MGMT* was generally observed in the mutants, even when their *MGMT* mRNA expression levels were low. The *MGMT* protein was shown to be degraded via the ubiquitin-proteasome pathway (35, 36). Therefore, *USP8* may play a role in the deubiquitination of *MGMT*. In this scenario, *MGMT* degradation is inhibited when adenomas either abundantly express *USP8* or have *USP8* mutations, leading to positive *MGMT* protein expression. However, elevated *MGMT* mRNA expression may be due to a *USP8* mutation-triggered epiphenomenon.

The alkylating compound, temozolomide, recently demonstrated a significant response against aggressive pituitary adenomas and carcinomas (19, 37, 38). A recent relatively large cohort study ($n=24$) (19) demonstrated that immunohistochemically low *MGMT* expression predicts a better response to temozolomide. In our series of CD, most CCA exhibited immunohistochemically negative *MGMT* expression, which was consistent with our previous findings (21). Although aggressive tumors including CCA are not good therapeutic candidates for pasireotide, such patients may have a better response to temozolomide.

Several studies have argued that an immunohistochemical *MGMT* expression analysis is not clinically useful in predicting tumor responses to temozolomide

therapy (20, 37, 38). One possible explanation of the controversy is the acquisition of temozolomide resistance due to the loss of MSH6 expression during temozolomide treatments (19, 20, 39). In our subjects, most patients (58 out of 60) were immunopositive for MSH6. Therefore, care is needed regarding the loss of MSH6 in acquiring temozolomide resistance after starting temozolomide treatments.

In summary, *USP8* mutations were frequently detected in females and smaller tumors. Microadenomas that strongly express *POMC* were common among the mutated tumors, which may provide an insight into the mechanisms by which microadenomas secrete excess ACTH in order to present overt CD. The frequency of *USP8* mutations was also significantly greater in tumors expressing SSTR5, suggesting favorable responses to the somatostatin analog pasireotide. In contrast, non-mutated aggressive tumors such as CCA may respond better to the alkylating agent temozolomide because of their significantly weak expression of *MGMT*. Although EGFR is theoretically activated in *USP8* mutant adenomas, we failed to demonstrate this *in vivo*. Further studies will be necessary to clarify this issue.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-15-0689>.

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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