USP8 mutations in corticotroph adenomas determine a distinct gene expression profile irrespective of functional tumour status

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Abstract

Objective: Pituitary corticotroph adenomas commonly cause Cushing's disease (CD) but part of these tumours are hormonally inactive (silent corticotroph adenomas, SCA). *USP8* mutations are well-known driver mutations in corticotrophinomas. Differences in transcriptomic profiles between functioning and silent tumours or tumours with different *USP8* status have not been investigated.

Design and methods: Forty-eight patients (28 CD, 20 SCA) were screened for *USP8* mutations with Sanger sequencing. Twenty-four patients were included in transcriptomic profiling with Ampliseq Transcriptome Human Gene Expression Core Panel. The entire patients group was included in qRT-PCR analysis of selected genes expression. Immunohistochemistry was used for visualization of selected protein.

Results: We found *USP8* mutation in 15 patients with CD and 4 SCAs. *USP8* mutations determine molecular profile of the tumours as showed by hierarchical clustering and identification of 1648 genes differentially expressed in *USP8*-mutated and *USP8*-wild-type tumours. Mutations affect many molecular pathways as observed in Gene Set Enrichment analysis. *USP8*-mutated adenomas showed higher level of *POMC*, *CDC25A*, *MAPK4* but lower level of *CCND2*, *CDK6*, *CDKN1B* than *USP8*-wt tumours. Eighty-seven genes differentially expressed between CD-related adenomas and SCAs were found, including those involved in cell signalling (*GLI2*, *DLC1*, *TBX2*, *RASSF6*), cell adhesion (*GJA1*, *CDH6*), ion transport (*KCNN4*, *KCNJ5*) and GABA signalling (*GABBR2*, *GABRD*).

Conclusion: *USP8* mutations occur in functioning and silent corticotrophinomas. They have pleiotropic effect, not limited to EGFR signalling, and affect expression levels of many genes involved in different pathways. Expression of GABA-related genes *GABBR2*, *GNAL*, *GABARD* and *KCNJ5* correspond to functional status of the tumours.

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Introduction

Pituitary tumours represent about 10–20% of all intracranial neoplasms and over 90% of tumours are located in the *sella turcica*; their prevalence in the population is estimated to be approximately 1/1000. These

tumours may arise from different kinds of secretory cells of pituitary gland, and therefore, they are characterized by overproduction of certain hormones which determine the clinical manifestation of the disease.

Corticotroph adenomas commonly cause ACTH-dependent Cushing's disease (CD) a severe, life-threatening disorder. However, a proportion of tumours originating from corticotrophic pituitary cells are hormonally non-functioning (1). They do not affect hormonal balance, do not cause CD symptoms and are classified as silent corticotroph adenomas (SCAs). Similar to most patients with non-functioning pituitary adenomas (NFPAs), SCA patients are diagnosed due to neurological symptoms (for example visual impairment) when tumours reach macroadenoma size (>10 mm). Corticotrophic nature of the tumours is confirmed in histopathological examination based on immunohistochemical staining. SCAs are commonly diagnosed at macroadenoma stage and represent one of the most aggressive subtypes of pituitary tumours characterized by invasive growth and high relapse rate (1).

Recently, notable progress in the understanding of molecular background of CD has been made (2) including the discovery of recurrent *USP8* hot spot mutation (3, 4, 5). In contrary, the biology of SCA still remains unexplored. The reason why some ACTH-producing adenomas (as confirmed by immunohistochemistry and electron microscopy) remain endocrinologically inactive is still unclear and the results of very few studies in this field were published (6, 7, 8, 9, 10, 11, 12, 13). Similarly, the molecular basis of aggressive growth of these tumours is very poorly understood (14, 15, 16, 17) and research on SCAs and CD-causing tumours using genome-wide molecular techniques is lacking.

The aim of the study was to compare gene expression profiles of functioning and SCAs in order to identify a biological mechanism contributing to distinct hormonal secretory activities. Since *USP8* is a well-recognized driver gene mutated in large proportion of corticotrophinomas, we intended to verify whether its mutations occur also in SCAs. Moreover, we intended to verify how *USP8* mutations affect transcriptomic profiles of CD and SCA.

Subjects and methods

Patients and samples

Pituitary tumour samples from 48 patients were collected during transsphenoidal surgery and fixed in formalin for routine diagnostic procedures. Diagnoses were based on WHO 2004 criteria. Archival FFPE tissue samples, including 28 samples from patients with CD and 20 samples of SCA from years 2013 to 2017 were chosen and reevaluated histopathologically.

The diagnosis of Cushing's syndrome/hypercortisolemia was based on standard hormonal criteria: increased urinary free cortisol (UFC) in three 24-h urine collections, disturbances of cortisol circadian rhythm, increased serum cortisol levels accompanied by increased or not suppressed plasma ACTH levels at 8:00 h, and a lack of suppression of serum cortisol levels to <1.8 µg/dL during an overnight dexamethasone suppression test (1 mg at midnight). The pituitary aetiology of CD was confirmed based on the serum cortisol levels or UFC suppression <50% with a high-dose dexamethasone suppression test (2 mg q.i.d. for 48 h) or a positive result of a corticotrophin-releasing hormone stimulation test (100 mg i.v.) and positive pituitary magnetic resonance imaging. In the group of SCAs, none of the patients had any evidence of hypercortisolemia based on clinical signs and symptoms as well as basic laboratory tests. ACTH levels were assessed using IRMA (ELSA-ACTH, CIS Bio International, Gif-sur-Yvette Cedex, France). The analytical sensitivity was 2 pg/mL (reference range: 10–60 pg/mL). Serum cortisol concentrations were determined by the Elecsys 2010 electrochemiluminescence immunoassay (Roche Diagnostics). Analytical sensitivity of the assay was 0.02 µg/dL (reference range: 6.2–19.4 µg/dL). UFC was determined after extraction (liquid/liquid with dichloromethane) by electrochemiluminescence immunoassay (Elecsys 2010, Roche Diagnostics) – reference range: 4.3–176 µg/24 h.

Characteristics of the patients are presented in Table 1. The study was approved by the Local Ethics Committee of Maria Skłodowska-Curie Institute – Oncology Center in Warsaw, Poland. Each patient provided informed consent for the use of tissue samples for scientific purposes. The content of tumour tissue in each FFPE sample ranged between 80 and 100% (median 99%), as described in detail in Supplementary Table 1 (see section on supplementary data given at the end of this article).

DNA and total RNA from FFPE samples was isolated using RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Thermo Fisher Scientific) and measured using NanoDrop 2000 (Thermo Fisher Scientific). The samples were stored at –70°C.

Assessment of *USP8* mutation status

The presence of point mutation at *USP8* hot spot (exon 14) was determined using Sanger sequencing. DNA was PCR amplified with FastStart Taq DNA Polymerase (Roche Diagnostics) using GeneAmp 9700 PCR system (Applied Biosystems). The PCR product was purified using ExoStar

Table 1 Patients' characteristics.

	CD	SCA
Number of patients	28	20
Age (years; median (range))	43 (23–76)	53 (23–77)
Gender		
Male	3	9
Female	25	11
Ultrastructural characteristics		
Sparsely granulated	9	12
Densely granulated	19	8
KNOSP grade		
0	5	2
1	12	9
2	4	5
3	3	2
4	4	2
Tumour size		
Macroadenoma	17	20
Microadenoma	11	0
<i>USP8</i> mutation	11	4

(GE Healthcare Life Sciences), labelled with BigDye Terminator v.3.1 (Applied Biosystems) according to the manufacturer's instructions and analysed by capillary electrophoresis with the ABI PRISM 3300 Genetic Analyzer (Applied Biosystems). PCR primers sequences are presented in Supplementary Table 2. NM_005154.5 (NP_005145.3) sequence was used as reference.

High-throughput transcriptome profiling with next-generation sequencing

RNA isolated from FFPE samples was used for amplicon-based library preparation and ion semiconductor sequencing. Human transcriptome sequencing library was prepared using the Ion AmpliSeq™ Transcriptome Human Gene Expression Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Briefly, 100 ng of total RNA was subjected to multiplex amplification with Ion AmpliSeq Transcriptome Human Gene Expression Core Panel (Thermo Fisher Scientific). Primer sequences of amplicons were digested and adapters were ligated to the amplicons. The samples were then purified using Agencourt AMPure XP beads (Beckman Coulter) and stored at –20°C for further processing. The concentration and DNA fragment length of each library was determined using Agilent High Sensitivity DNA Analysis Kits on a Bioanalyzer 2100 (Agilent). Each library was diluted to ~55 pM prior to template preparation. Up to seven barcoded libraries were subjected to automated template preparation with the Ion PI Hi-Q Chef Kit on the Ion Chef Instrument, which performs emulsion PCR on Ion Sphere Particles, followed by particle recovery and template

loading on a PI chip. Samples were sequenced in the Ion Proton instrument on a PI chip using the sequencing reagents (Ion PI™ Hi-Q™ Sequencing 200 Kit) provided as part of the Ion PI™ Hi-Q™ Chef Kit according to the manufacturer's instructions (Thermo Fisher Scientific).

All the steps of data analysis were performed using Bioconductor packages in R programming language environment. The raw un-normalized count matrix was generated from BAM files using Genomic Alignments package (18). Then it was imported to DESeq2 which performed the necessary normalization and calculated estimates of dispersion (19), and then low-expression gene filtration was applied (the genes with at least five sequencing reads in at least half of the samples were included) and subsequent analysis of differentially expressed genes between groups by use of discrete distributions and negative binomial generalized linear models was performed. Fold change of expression (FC), calculated as read counts ratio *USP8*-mutated (*USP8*mut)/*USP8*-wild type (*USP8*wt) or CD/SCA was used as a measure of expression difference between the groups of samples. Differentially expressed genes (DEGs) were defined as those with adjusted *P* value <0.05. DEGs with FC >2.5 or FC <0.4 were used for Gene Set Enrichment analysis (GSEA).

Gene expression assessment using qRT-PCR

Expression levels of the selected genes were assessed using qRT-PCR. One microgram of each FFPE tissue-derived RNA sample was subjected to reverse transcription using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). qRT-PCR was carried out using a 384-well format and the 7900HT Fast Real-Time PCR System (Applied Biosystems) with Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) in a volume of 5 µL, containing 2.25 pmol of each primer. The samples were amplified in triplicates. *GAPDH* was used as reference gene. Standard curves, based on amplification of known cDNA template concentrations, were used for determining PCR efficiency. Delta Ct method was used to calculate relative expression level. PCR primers' sequences are presented in Supplementary Table 2. PCR nondetects were used in calculation of relative gene expression level with the use of *nondetects* R package (20).

Immunohistochemical staining

Immunohistochemical staining (IHC) was performed on 4-µm FFPE tissue sections from 24 samples (12 SCA and

12 CD tumours) using the Envision Detection System (Dako). Sections were deparaffinized with xylene and rehydrated in a series of ethanol solutions of decreasing concentration. Heat-induced epitope retrieval was carried out in a Target Retrieval Solution pH 9 (Dako) in a 96°C water bath, for 20 min. After cooling the retrieval solutions for 25 min at room temperature, the slides were treated for 5 min with a Blocker of Endogenous Peroxidase (Dako). Slides were incubated overnight at 4°C with polyclonal antibody anti-KCNJ5 (PA5-53594, Thermo Fisher Scientific) (dilution 1:50) and subsequently labelled with the Envision Detection System (Dako). The colour reaction product was developed with 3,3'-diaminobenzidine tetrahydrochloride (Dako) as a substrate, and nuclear contrast was achieved with hematoxylin counterstaining. Analysis of immunohistochemical reactivity was performed by calculating H-score, which combines information on both reaction intensity (scored from 0 to 3) and number of the cells with a given intensity. Previously reported formula was used for quantification (21). Scoring results were analysed as continuous variables.

Statistical analysis

Quantitative continuous variables were analysed using the two-sided Mann–Whitney *U* test. Two-sided Fisher's exact test was used for the analysis of proportions. A significance threshold level $\alpha=0.05$ was used. Data were analysed and visualized using GraphPad Prism (GraphPad Software). Hierarchical clustering using the values for all the genes that passed the filtration was done using hclust function, (stats R package). Complete-linkage clustering with Euclidean distance as similarity metrics was used. EnrichR tool was used for Gene Set Enrichment (GSE) analysis (22). The GSE terms meeting the criterion of adjusted $P<0.05$ were considered as significantly enriched. Cluster 3.0 and TreeView 1.6 software (Stanford University School of Medicine, Stanford, CA, USA) were used for heat map preparation.

Results

Prevalence of *USP8* mutation in patients

Hot spot mutations in exon 14 of *USP8* were found in 15 patients including 11/28 (39.2%) of CD adenomas and 4/20 (20%) SCAs. The mutations were missense variants: p.Pro720Arg (five patients), p.Pro720Gln (one patient), p.Ser718Pro (two patients) and in-frame

deletion p.Ser718del (seven patients). All the mutations were heterozygous. The results of Sanger sequencing are presented in Supplementary Fig. 1. The observed prevalence of mutations was higher in CD than in SCA patients, but the difference did not reach significance threshold level ($P=0.2122$). No difference in the frequency of *USP8* mutations was observed in patients stratified according to results of ultrastructural examination of the tumours (*USP8* mutation was detected in 10/27 of densely granulated and in 5/21 of sparsely granulated adenomas, $P=0.366$) and sex of the patients (mutation was detected in 1/12 man and 14/36 women; $P=0.0729$). No difference in age of the *USP8*mut and *USP8*wt patients was found (mean age 43.8 vs 50.8, respectively; $P=0.1220$).

Effect of *USP8* mutation on gene expression profile

Twenty-four patients including 12 CD patients (6 *USP8*mut and 6 *USP8*wt) as well as 12 SCA patients (4 *USP8*mut and 8 *USP8*wt) were subjected to gene expression profiling with the use of Ion AmpliSeq Transcriptome Human Gene Expression Core Panel. Following data filtration and normalization, 17 550 human genes were subjected to analysis that involved hierarchical clustering and identification of genes differentially expressed between functioning and silent corticotrophinomas as well as between *USP8*mut and *USP8*wt tumours. PCA analysis performed on read counts normalized for library size showed four outlier samples (Supplementary Fig. 2). These samples were excluded from the analysis to avoid technical bias. The data were deposited in NCBI's Gene Expression Omnibus and are accessible through GSE132982 accession number.

Hierarchical clustering of samples based on whole transcriptional data revealed that *USP8*mut tumours represent a separate gene expression cluster, irrespective of their endocrinological status (Fig. 1A). The predominant role of *USP8* mutations in defining the phenotype of corticotroph adenomas was confirmed by identification of 1648 DEGs differentiating *USP8*mut and *USP8*wt tumours (adjusted P value <0.05). These DEGs, including 595 genes with FC >2.5 or FC <0.4 , are presented in Fig. 1B and listed in Supplementary Table 3. Using Gene Ontology (GO) analysis as well as Panther and Reactome Pathways databases with EnrichR tool (22), we sought the molecular pathways represented by these 595 DEGs by GSE analysis. We identified 11 GO terms enriched with DEGs related to few general processes: nervous system development and functioning, nucleosome organization, cell-cell adhesion and regulation of cell

Figure 1

Results of transcriptomic profiling of corticotroph tumours with regards to *USP8* mutation and functional status. (A) Hierarchical clustering of tumour samples based on the transcriptional profile (17 550 genes). (B) Volcano plots presenting distribution of DEGs identified in comparisons of *USP8*mut vs *USP8*wt tumours as well as SCAs vs functioning corticotrophinomas. (C) Expression of genes differentially expressed in SCAs and adenomas causing Cushing's disease with FC >2.5 or FC <0.4.

proliferation. Two Panther pathways were identified as significantly enriched: cadherin signalling and Wnt signalling pathways. Seventy-three reactome pathways were identified as significantly enriched. Most of the pathways were identified due to the high representation of genes encoding replication-dependent histone variants that are expressed at significantly lower level in *USP8*mut tumours. Top GSE results are presented in Table 2, while details are shown in Supplementary Table 3.

Gene expression in functioning and silent corticotrophinomas

When comparing silent and functional corticotrophinomas, eighty-seven differentially expressed genes were found (Supplementary Table 4) including 66

genes with FC >2.5 or FC <0.4 that are presented in Fig. 2B. These genes encode functionally diverse proteins that play a role in different cellular processes including cell signalling (*GLI2*, *DLC1*, *TBX2*, *RASSF6*, *GNAL*, *RASGRP2*, *PDGFRB*, *GABBR2*), cell adhesion (*GJA1*, *CDH6*), ion channels functioning (*KCNN4*, *KCNJ5*, *GABRD*) and others. The highest fold change of expression was observed for *KCNJ5* (most upregulated in SCAs) and *GABBR2* (most downregulated in SCAs) genes that both belong to a GABAB receptor pathway according to Reactome db. GSE analysis with Reactome database showed GABAB receptor activation (Reactome pathways R-HSA-991365 and R-HSA-977444) as the only pathway significantly enriched for the differentially expressed genes (Table 2 and Supplementary Table 4). Three DEGs were annotated to these pathways: *GABBR2*, *KCNJ5* and *GNAL*. No significant enrichment was

Table 2 The results of Gene Set Enrichment analysis for genes differentially expressed in USP8-mutated and USP8 wild-type tumours.

	Term	Overlap	Adjusted P
GSE analysis for genes differentially expressed in USP8-mutated and USP8 wt patients			
GO molecular processes			
1	Synapse assembly (GO:0007416)	16/64	<0.0001
2	Nucleosome assembly (GO:0006334)	10/61	0.0092
3	Anterograde trans-synaptic signalling (GO:0098916)	21/241	0.0092
4	Chromatin assembly (GO:0031497)	10/66	0.0096
5	Nervous system development (GO:0007399)	31/456	0.0096
6	Cell-cell adhesion via plasma-membrane adhesion molecules (GO:0098742)	15/143	0.0096
7	Chemical synaptic transmission (GO:0007268)	22/290	0.0208
8	Nucleosome organization (GO:0034728)	10/80	0.0381
9	Negative regulation of epithelial cell proliferation (GO:0050680)	9/67	0.0419
10	Extracellular matrix organization (GO:0030198)	18/230	0.0453
Panther pathways			
1	Cadherin signalling pathway_Homo sapiens_P00012	17/150	0.0002
2	Wnt signalling pathway_Homo sapiens_P00057	20/278	0.0088
Reactome pathways			
1	RNA Polymerase I Promoter Opening_Homo sapiens_R-HSA-73728	10/30	<0.0001
2	Packaging Of Telomere Ends_Homo sapiens_R-HSA-171306	10/31	<0.0001
3	DNA methylation_Homo sapiens_R-HSA-5334118	10/32	<0.0001
4	Condensation of Prophase Chromosomes_Homo sapiens_R-HSA-2299718	11/41	<0.0001
5	DNA Damage/Telomere Stress Induced Senescence_Homo sapiens_R-HSA-2559586	13/59	<0.0001
6	Activated PKN1 stimulates transcription of AR (androgen receptor) regulated genes KLK2 and KLK3_Homo sapiens_R-HSA-5625886	10/34	<0.0001
7	SIRT1 negatively regulates rRNA Expression_Homo sapiens_R-HSA-427359	10/35	<0.0001
8	Meiotic recombination_Homo sapiens_R-HSA-912446	12/54	<0.0001
9	PRC2 methylates histones and DNA_Homo sapiens_R-HSA-212300	10/40	<0.0001
10	Cellular Senescence_Homo sapiens_R-HSA-2559583	19/161	<0.0001
GSE analysis for genes differentially expressed in SCAs and functioning corticotrophinomas			
Reactome pathways			
1	Activation of GABAB receptors_Homo sapiens_R-HSA-991365	3/39	0.0003
2	GABA B receptor activation_Homo sapiens_R-HSA-977444	3/39	0.0003

Up to top ten pathways are presented.

found in GSE analysis based on GO biological processes and Panther pathways.

Evaluation of the expression level of selected genes with qRT-PCR

The expression levels of 12 genes were assessed in 48 adenoma tumour samples with qRT-PCR. This set included *POMC* gene not included in Ion AmpliSeq Transcriptome Human Gene Expression Core Panel, *USP8*, *EGFR*, selected genes identified as differentially expressed in *USP8mut* and *USP8wt* tumours (*CCND2*, *CDK6*, *CDKN1B*, *CDC25A* and *MAPK4*) as well as the genes related to GABA

signalling (*KCNJ5*, *GABBR2*, *GNAL* and *GABARD*) (found as differentially expressed in SCA and CD patients).

USP8mut tumours revealed slightly higher *POMC* expression than *USP8wt* tumours (Fig. 2A), while no difference in the expression level of *EGFR* and *USP8* was found between these tumours (Supplementary Fig. 3). Higher levels of *CCND2*, *CDK6* and *CDKN1B* were observed in *USP8wt* tumours, *CDC25A* and *MAPK4* expression level were higher in samples with *USP8* mutation.

POMC expression was found lowered in non-functioning adenomas as compared to CD; however, decreased expression was observed predominantly in *USP8wt* tumours.

Figure 2

The results of gene expression measurements with qRT-PCR. (A) Comparison of gene expression levels in corticotroph tumours with *USP8* mutation and wild-type corticotroph tumours. (B) Comparison of gene expression levels in SCA and functioning corticotroph tumours with regard to *USP8* mutation status. (C) Spearman correlation between *GABBR2* and *KCNJ5* expression level.

The expression of *KCNJ5* was higher in SCA patients than in patients with Cushing's disease, whereas mRNA levels of *GABBR2*, *GNAL* and *GABRD* were higher in CD patients (Fig. 2B), in line with the results of NGS-based

transcriptomic profiling. Since *KCNJ5*, *GABBR2* and *GNAL* encode proteins involved in the same pathway, we examined the correlation between these genes expression levels. This analysis showed a negative correlation between

KCNJ5 and *GABBR2* (Spearman $R=-0.37$; $P=0.0095$) as shown in Fig. 2C.

Immunohistochemical evaluation of *KCNJ5* protein expression

IHC was performed on 22 FFPE samples (11 SCA and 11 CD tumours). Both membrane and cytoplasmic immunoreactivity was observed in 7/11 SCA samples and 4/11 adenomas causing CD (differences in the proportions did not reach significance threshold level). Nine tissue samples revealed only cytoplasmic immunoreactivity. The examples of tissue staining are presented in Fig. 3. H-score was calculated for all 22 patients and comparison of silent and functioning corticotrophinomas was performed. SCA samples showed higher immunostaining reactivity score as compared to functioning tumours with mean H-core 146 and 82.2 in SCA and CD patients, respectively ($P=0.0104$; Fig. 3). Positive correlation between H-score and expression level of *KCNJ5* measured with qRT-PCR was observed (Spearman $R=0.447$; $P=0.0368$).

Discussion

The frequency of *USP8* mutations in CD patients observed in our study is similar to previously reported results where the mutations were identified in about 30–40% of the tumours (3, 5, 23, 24). However, both lower and higher mutations frequencies were also reported in other cohorts (4, 25, 26). In our study, all the identified *USP8* mutations were single amino acid substitutions or single codon deletion in hot spot region in exon 14. All the alterations were also identified previously (4, 5, 23, 24, 27).

Interestingly, in contrary to previous studies we also found *USP8* mutations in SCA patients (4/20). In the published reports (5, 26) no mutations were observed in non-functioning corticotroph tumours, however, a smaller number of SCA samples (11 and 2 samples) were included. After careful verification of clinical data to exclude misdiagnosis of these patients, we confirmed all the cases to meet criteria for SCAs. Regardless of diagnostic criteria, the biological differences between SCAs and functioning corticotroph tumours are still unclear (1).

A Functioning Corticotroph Tumors

B Silent Corticotroph Tumors

Figure 3

The results of immunohistochemical *KCNJ5* detection in tissue samples. (A) The examples of immunostaining in functioning and silent corticotroph tumours. (B) The results of immunohistochemical staining evaluation. Comparison of H-score values in silent and functioning corticotroph tumours. A full colour version of this figure is available at <https://doi.org/10.1530/EJE-19-0194>.

Both tumour types develop from the same pituitary cell lineage and the evidence of SCA to CD conversion and vice versa was also reported (8, 28). Our results may suggest that *USP8*mut SCAs may have different molecular profile of gene expression than *USP8*wt SCAs, including higher *POMC* expression. However, this issue was not investigated due to a low number of patients.

The gene expression profiling of corticotroph adenomas clearly showed that *USP8* mutations strongly affect molecular profile of tumours regardless of functional status. Hierarchical clustering based on whole transcriptomic data showed that *USP8*mut samples represent a separate cluster. In terms of genes expression, these samples are more similar to each other than samples from functional groups (silent and functioning corticotrophinomas). Presence of the mutations affects the expression of numerous genes involved in many different molecular processes and pathways. Processes related to cell-cell adhesion, cadherin signalling and Wnt signalling were found to be affected by *USP8* mutation status. These pathways were identified as significantly enriched due to the high representation of protocadherin-encoding genes and *FZD1* in DEGs.

A large number of different pathways in GSE analysis with reactome database were found due to high representation of genes encoding replication-dependent histone variants among DEGs (with lower expression in *USP8*mut patients). More than half of all histone-encoding genes (29/49) that passed the filtration in the analysis of NGS results are differentially expressed. Alteration in the expression of this class of genes causes imbalance in the composition of histones by affecting chromatin organization and functional status (29). This may lead to disturbances in various cellular processes. Deregulation of histone-encoding genes' expression was described in carcinogenesis (29).

Cell cycle regulators have been recognized as involved in development of corticotroph tumours (30). In line with this are our findings of identification of DEGs among known cancer-related genes involved in the regulation of cell proliferation. Negative regulation of epithelial cell proliferation was identified among the enriched GO processes in GSE analysis due to differential expression of genes including *CDK6*, *TGFB2*, *SOX2*, *SOX9*, and *SNAI2*. As also further confirmed by qPCR evaluation, *CCND2* (encoding cyclin D2) and *CDK6* expression was higher in *USP8*wt tumours, suggesting increased cyclin D signalling in these tumours. In *USP8*mut tumours we observed low level of *CDKN1B* (encoding p27/Kip1 inhibitor) and high expression of *CDC25A*, a known cell cycle-related

oncogene (31). Correspondingly, a difference in p27/Kip1 protein level in mutated and wild-type corticotrophinoma patients was reported recently (27). *MAPK4* (encoding ERK4) with higher expression in *USP8*mut tumours is atypical MAP kinase that was found to be overexpressed in human cancers. Its overexpression results in the increased cell proliferation through stimulation of AKT/mTOR signalling (32).

Mutations in *USP8* hot spot occur in the region involved in binding 14-3-3 proteins and thus impair interactions between *USP8* and 14-3-3 proteins (33). As a consequence, the activity of *USP8* is enhanced leading to deubiquitination of its substrates and preventing their proteasomal degradation. This mechanism was clearly shown *in vitro* providing the explanation of the sustained EGFR signalling in corticotroph tumours with *USP8* mutations (3, 4). However, there is also evidence that the level of other proteins associated with tumour development such as *MET1* (34) and *Cdt2* (35) may be sustained with this mechanism. Regulatory role of *USP8* in preventing degradation of *VEGFR2* (36), smoothened (*SMO*) (37), frizzled (*FZD*) (38) and *Nrdp1* (39) was also reported. The large number of DEGs and the enriched pathways in comparison of *USP8*-mutated and wild-type tumours suggest that the mutation exerts a pleiotropic biological effect not limited to EGFR pathway that it is manifested by deregulation of many proteins. When comparing *USP8*mut and *USP8*wt patients we did not observe difference in *USP8* and *EGFR* expression levels, as also reported previously (4, 24). We also did not find EGFR-related pathways as significantly enriched for DEGs in GSE analysis.

According to our study and previous research (4, 24) *USP8*mut tumours show higher *POMC* expression, which is concordant with the reported increase in ACTH expression caused by enhanced activity of *POMC* promoter in *USP8*mut tumours (3).

We have paid special attention to comparison of gene expression profiles of SCAs and functioning corticotroph tumours causing CD. The biological differences between functioning and non-functioning corticotroph tumours have been investigated in very few studies. Intriguingly, the cause of the silent nature of SCAs remains elusive. Unexpectedly, our results show that the functionally active and silent corticotrophinomas differ only slightly in terms of genes expression with only 66 DEGs (with FC >2.5) found in this comparison. The identified genes are involved in cell signalling (*GLI2*, *DLC1*, *TBX2*, *RASSF6*), cell adhesion (*GJA1*, *CDH6*) or ion channels functioning (*KCNN4*, *KCNJ5*). We did not find significant differences in

the expression of genes related to ACTH release including prohormone convertase 1/3 gene (9, 10, 12), *SSTR1*, *SSTR2*, *CRHR1*, *AVPR1B* (*V1BR*), *HSD11B2* (7, 12) or genes related to differentiation of pituitary cells *NEUROD1* and *TBX19* (*TPIT*) that have been reported previously as differentially expressed in SCAs and functioning corticotrophinomas (7, 12).

The physiological activity of pituitary cells is regulated by changes in membrane potential. Secretory activity of corticotroph cells is provoked by membrane depolarization associated with the opening of Ca^{2+} channels and the influx of these ions into the cell (40). Therefore, the genes involved in electrical excitability of cells are of a special interest when comparing SCA and functioning tumours. Indeed, genes encoding ion channels (*KCNJ5*, *KCNN4*, *GABRD*), as well as other genes involved in regulation of membrane potential (*GABBR2*, *GNAL*, *GJA1* – connexin 43) were found differentially expressed.

KCNJ5 was identified as a DEG with the highest fold change of expression between SCAs and CD adenomas. It encodes KIR3.4 (*GIRK4*) protein, which is a G-protein-activated inward rectifier potassium channel. KIR3 family channels are involved in stabilization of resting membrane potential (RMP) and prevent membrane hyperpolarization. Blocking K^{+} channels results in membrane depolarization and increase in Ca^{2+} concentration that triggers ACTH secretion in corticotroph cells (41). KIR channels were shown to be involved in maintaining RMP in corticotroph cells and their inhibition contributes to firing activation potential upon CRH stimulation (42).

Four KIR3 channels have been identified in humans. KIR3-encoding genes are mostly expressed in brain and pituitary, with exception of KIR3.4 (*KCNJ5*) that is mostly expressed in adrenal gland and pituitary (based on GtEX database) (43). In pituitary, *KCNJ5* has the highest expression of all KIR3-encoding genes (based on GtEX database). In adrenal gland KIR3.4 seems to play a pivotal role in aldosterone secretion. Somatic *KCNJ5* mutations are found in aldosterone-producing adenomas, while germline mutations of this gene cause familial hyperaldosteronism type 3. *KCNJ5* mutations result in loss of selectivity for K^{+} , causing Na^{+} influx, calcium mobilization, increased depolarization, and hyperaldosteronism (44). KIR3 channels are downstream effectors of G-protein-coupled receptors (45). As observed in corticotroph AtT-20 cells, stimulation of somatostatin receptor (46, 47) and dopamine receptors (48) results in activation of KIR channels. Since KIR channels are involved in a negative regulation of ACTH secretion,

high expression of *KCNJ5* in SCAs can be considered as functionally related to inactive nature of these tumours.

When GSE method was applied for the analysis of genes differentially expressed between SCAs and functioning corticotrophinomas, the enrichment in the processes of activation of GABA receptor B (*GABARB*) was the only pathway found in Reactome database. This Reactome pathway includes three DEGs: *KCNJ5* (described above) as well as *GABBR2* and *GNAL*, both with higher expression in functioning tumours. Additionally, CD-causing tumours showed also higher expression of another gene related to GABA signalling – *GABRD*. In anterior pituitary GABA acts in a paracrine and autocrine manner, and it stimulates some functional subtypes of the cells including corticotrophs, while it has inhibitory effect on other ones (49, 50, 51). Accordingly to this general role of GABA, we observed the higher expression of genes encoding subunits of GABA receptors B (*GABBR2*) and A (*GABRD*) in functioning corticotroph tumours than in SCAs. The possible functional consequences of these results are unclear. *GABARB* subunits are expressed in corticotroph cells (50, 51), but the available data indicate that this receptor does not play a role in ACTH release (49, 51, 52). In corticotroph cells, hormone secretion is mediated by stimulating GABA receptor A (*GABARA*) rather than *GABARB*, as the cells release ACTH upon stimulation with muscimol, and not with baclofen (46, 48). On the other hand, *GABARA* receptor is a large pentameric ligand-gated ion channel consisting of five subunits of different types ($\alpha 1$ -6, $\beta 1$ -3, $\gamma 1$ -3, δ , ϵ , π and θ) and only the gene encoding subunit δ (*GABRD*) is expressed at higher level in functioning adenomas than in SCAs. It is not clear how overexpression of one subunit only would affect receptor function. It was shown that stimulation of δ subunit with specific agonist enhances secretory activity of pituitary gonadotrophs (53), but no data regarding the role of this subunit in corticotroph cells are available.

According to GSE analysis with Reactome, *KCNJ5* and *GNAL* play a role in *GABARB* signalling. However, considering the reports of no influence of *GABARB* stimulation on ACTH release, it is fairly probable that *KCNJ5* and *GNAL* (encoding G-protein subunit alpha) act as effectors for other G-protein-coupled receptors. The role of GABA signalling has been most extensively studied in adult neurons, where KIR channels are one of the effectors for *GABAB* receptor (54). In corticotroph tumours we observed the inverse correlation of expression of *GABBR2* and *KCNJ5* that suggest the opposite role of these genes.

In summary, our results show that *USP8* hot spot mutations occur in corticotroph adenomas causing CD as

well as in SCAs albeit with low frequency. *USP8* mutations determine the molecular profile of the tumour. They have a pleiotropic effect affecting the expression levels of numerous genes involved in different molecular processes. Expression levels of relatively low number of genes were found to distinguish SCAs and CD-causing tumours. Our findings suggest that high *KCNJ5* expression may be involved in lowering electrical excitability of the cells in SCA, while high expression levels of genes involved in GABA signalling may favour ACTH hypersecretion in tumours causing CD.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/EJE-19-0194>.

Declaration of interest

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

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