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ORIGINAL ARTICLE

# Ubiquitin-specific Protease 8 Gene Expression in Sporadic Pituitary Adenomas

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## Abstrac

**OBJECTIVES:** In sporadic pituitary adenomas, the role of Ubiquitin-specific protease 8 (USP8) is not clearly defined. Mutations in USP8 gene are known to influence formation of the corticotroph adenomas. However, it has not been clarified whether changes in expression of USP8 have an impact on other pituitary adenomas or not. In this study we addressed the changes in USP8 gene expression levels in pituitary adenomas (PA) relative to non-adenomatous brain tissue.

**MATERIAL AND METHODS:** USP8 gene expression analysis was performed on a total of 43 tissue samples from human pituitary adenomas and on 16 tissue samples from non-pituitary brain tissues (control group). Adenomatous tissues and control tissues were assessed for quantification of RNA expression of USP8. The levels of USP8 gene expression were determined relative to those in control group.

**RESULTS:** Overall, the USP8 gene expression levels in PA were 3.7 times higher than the control brain tissues (CBT) ($p$=0.002). However, after stratification, only the USP8 in the secretory PA were higher than CBT($p$=0.002).

**CONCLUSIONS:** Present findings support that USP8 gene expression levels may contribute to pitutary tumorigenesis and hormonogenesis.

**Abbreviations:**

| | |
|---|---|
| ACTB | - Actin beta |
| ACTH | - Adrenocorticotropic hormone |
| cDNA | - Complementary DNA |
| CBT | - Control brain tissues |
| EGF | - Epithelial growth factor |
| EGFR | - Epidermal growth factor receptor |
| GNAS | - Guanine nucleotide binding protein, alpha stimulating |
| MRI | - Magnetic resonance imaging |
| PA | - Pituitary adenomas |
| RT-qPCR | - Real-time quantitative PCR |
| RT | - Reverse transcription |
| Ct | - Threshold cycle |
| USP8 | - Ubiquitin-specific protease 8 |

## INTRODUCTION

Pituitary tumors are among common intracranial neoplasms with increasing prevalence of 14-22 % based on post-mortem and radiologic series (Ezzat *et al.* 2004). Although the majority of them demonstrate benign characteristics, localisation, size and hormonal activity of the tumor create a significant clinical impact and burden on patients.

Most of the pituitary tumors are known to be sporadic (Caimari & Korbonits, 2016). Genetic mechanisms contributing to pituitary tumorigenesis have not been completely unraveled, yet. To date, only a limited number of somatic mutations, including those of guanine nucleotide binding protein, alpha stimulating (GNAS) and ubiquitin-specific protease 8 (USP8) genes, have been identified in pituitary adenomas (Bi *et al.* 2017, Song *et al.* 2016).

USP8 which is a deubiquitinase involved in various cellular processes, and altered USP8 gene expression has been reported in various cancers such as cholangiocarcinoma, breast and gastric cancers (Sun *et al.* 2020, Jing *et al.* 2020, Shin *et al.* 2020, Qiu *et al.* 2018, Yan *et al.* 2018). This alteration in gene expression, hence the USP8 enzyme level, has been considered as a prominent mechanism for tumorigenesis and tumor progression (Sun *et al.* 2020, Hussain *et al.* 2009, Byun *et al.* 2013). USP8 acts by removing conjugated ubiquitin from proteins and decreasing protein degradation. It is also involved in reversal of ubiquitination which results in downregulation of epidermal growth factor receptor (EGFR) , hence contributing to tumorigenesis (Kato *et al.* 2000, Gnesutta *et al.* 2001, Row *et al.* 2006, Mizuno *et al.* 2005, Alwan & van Leeuwen, 2207).

In human, the gene for USP8 is located on chromosome 15 (Giovanna & Martegan,2013). Mouse models demonstarted a substantial level of USP8 expression in the brain (Gnesutta *et al.* 2001). However, studies focusing on USP8 expression in normal human tissues are limited. Recent studies have revealed that USP8 gene had a low region-specificity, present in almost all human tissues (Uhlén *et al.* 2015, https://www.proteinatlas.org/ENSG00000138592-USP8). Multiple post-mortem human brain tissues highlighted a wide distribution and high expression of USP8 (https://www.proteinatlas.org/ENSG00000138592-USP8).

Although USP8 somatic mutations in corticotroph adenomas have been reported previously, its role in other types of pituitary adenomas has not yet determined. Herein, we aimed to investigate the USP8 expression levels in pituitary adenomas and their implications for clinical practice.

## MATERIAL AND METHODS

USP8 gene expression analysis was performed on a total of 43 tissue samples from human pituitary adenomas (7 non-secretory, 36 hormone secreting) and on 16 tissue samples from non-pituitary brain tissues (control group). The group with PA was stratified by their secretory status. Those with a hormone secretion or a positive immunohistochemical staining for any hormone were depicted as secretory PA, whereas those without secretion and with a negative immunohistochemical staining were depicted as non- secretory PA.Family history of a pituitary adenoma was excluded for each case in the study group. Preoperative basal pituitary hormone levels and pituitary magnetic resonance imaging (MRI) findings were also obtained for cases with pituitary adenoma. In the control group, absence of a pituitary mass was confirmed radiologically prior to surgery. Tissues from pituitary adenomas were obtained during the adenoma surgery, whereas control brain tissues (CBT) consisting of temporal lobe parenchyma were obtained from patients who underwent medial temporal sclerosis surgery. The ethical committee of the Bakirkoy Dr. Sadi Konuk Training and Research Hospital, approved the study and informed consents were provided for investigations.

### *RNA extraction and real-time quantitative PCR (RT-qPCR)*

Immediately after resection, the tissue samples were collected in tubes and frozen at –80 °C. Ribonucleic acid (RNA) was extracted from tissue and serum samples using a Trizol reagent (Invitrogen, Carlsbad, CA, USA) to obtain the total RNA, according to the manufacturer's recommendations. Complementary DNA (cDNA) was transcribed from 100 ng of total RNA using a High-Capacity cDNA Reverse Transcription (RT) Kit (Applied Biosystems, Foster City, CA, USA), in a total volume of 20 μL. RT master mix contained the following: 10 × RT buffer, 25 × dNTP (deoxynucleotide) mix (100 mM), 10 × RT random primers, MultiScribe™ reverse transcriptase, RNase inhibitor, and nuclease-free water. The RT reaction was performed in a Thermocycler (Eppendorf, Hamburg, Germany) in the following conditions: 5 min at 25 °C, followed by 60 min at 42 °C, then the samples were heated to 70 °C for 5 min. The relative expression of USP8 was assessed using TaqMan® probes (Applied Biosystems) for the studied gene (Hs00987105_g1), with actin beta (ACTB) (Hs99999903_m1) as the reference gene. The procedure was performed in an Applied Biosystems 7500 Fast Real-Time PCR System, for 40 cycles. The PCR mixture was as follows: cDNA (1–100 ng), 20 × TaqMan® Gene Expression Assay, 2 × TaqMan® Gene Expression Master Mix, and RNase-free water, in a total volume of 20 μL. Gene expression levels were quantified using 7500 Fast Real-Time Sequence detection system Software (Applied Biosystems, Foster City, CA). Gene expression was defined based on the threshold cycle (Ct), and ACTB was used as a reference gene that acts as an internal reference to normalize the RNA expression, which was calculated as $2-\Delta\Delta CT$.

**Tab. 1.** Demographic and hormonal characteristics of the group with pituitary adenoma (n=43)

| | NFA | Lactotroph | Somatotroph | Corticotroph | Gonadotroph |
|---|---|---|---|---|---|
| Age (year) | 46.7±14.2 | 48.7±18 | 46.2±10.9 | 43.8±18.4 | 56.6±14.2 |
| Gender (F/M,n) | 1/6 | 1/2 | 9/4 | 3/3 | 5/9 |
| GH (ng/ml) | 0.26 [0.1-0.3] | 2 [0.4-3.7] | 1.2 [1.2-3.3] | 0.22 [0.14-1.6] | 0.16 [0.1-0.4] |
| PRL (ng/ml) | 15.4 [9-24.9] | 12.6 [0.4-1771] | 3.9 [1.7-14.2] | 7.6 [2.4-18.3] | 16.1 [7.2-24.4] |
| ACTH (pg/ml) | 15.7 [10.4-61] | 27.8 [21.8-33.8] | 32.2 [8.3-42.9] | 46.5 [18.7-72.1] | 27.7 [14.9-33.6] |
| FSH (IU/ml)) | 7.2 [1.6-12.5] | 1.6 [1.2-2.9] | 4.5 [3.6-26.3] | 6.4 [3.2-11.1] | 5.4 [1.7-9.2] |
| LH (IU/ml)) | 3.7 [1.6-12.5] | 1.1 [0.8-3.1] | 6 [2.9-16.4] | 4.6 [2.9-5.8] | 2.9 [0.8-5.4] |
| TSH (mIU/ml) | 1 [0.6-1.5] | 1.8 [1.8-2.2] | 1.2 [0.8-1.5] | 1 [0.7-1.4] | 1.4 [0.8-2.3] |

ACTH: adrenocorticotropic hormone, FSH: follicle stimulating hormone, GH: growth hormone, LH: luteinizing hormone, NFA: nonfunctional adenoma, PRL: prolactine, TSH: thyroid stimulating hormone, F: female, M: male, N/A: not available.
¶ Data was expressed as median and IQR.

Data was statistically analyzed by SPSS 15.0 package program. The results are presented as medians and interquartile ranges [IQR]. The Kruskal-Wallis test was used to compare the medians between the multiple groups. The Mann-Whitney U test was used to compare two independent variables. Pearson's correlation coefficient was used for the calculation of associations between variables. The $\chi^2$ test was used when necessary. $p$ <0.05 was considered statistically significant.

## RESULTS

The group with PA was stratified by their status of secretion. Those with secretory PA were further stratified by their hormonal characteristics. Demographic information of the pituitary adenoma group and hormonal characteristics of the adenomas are represented in Table 1.

Overall, USP8 gene expression levels in pituitary adenomas (PA) were found to be 3.7 [IQR: 1.1-50.2] times higher than the levels in CBT ($p$=0.002) (figure 1). After stratification of the PA by their secretory status, USP8 gene expression levels in the secretory PA were higher than the levels in non-secretory PA. However, the difference was not statistically significant (for secretory PA: 4.2 [IQR: 1.2-50.6] and for non-secretory PA: 1.9 [IQR: 0.6-42.6], $p$=0.6). Levels of USP8 gene expression in the secretory PA were significantly higher in comparison to the levels in CBT ($p$=0.002). However, expression levels in non-secretory PA were not statistically different from those in CBT ($p$=0.09) (figure 2).

When the secretory PAs were further classified based on their hormone secretion, there was no significant difference between the USP8 expression levels of these subgroups (for follicle stimulating hormone/ luteinizing hormone secreting PA: 17.9 [IQR: 1-3.8], for

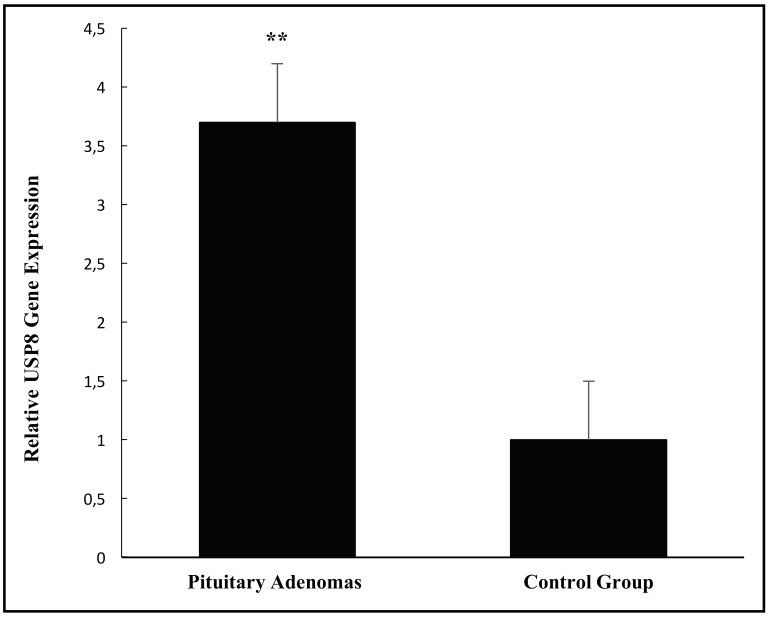


**Fig. 1.** Relative expression levels of USP8 (Ubiquitin-specific protease 8) in pituitary adenomas and non-adenomatous brain tissue (control group), ** $p$=0.002.

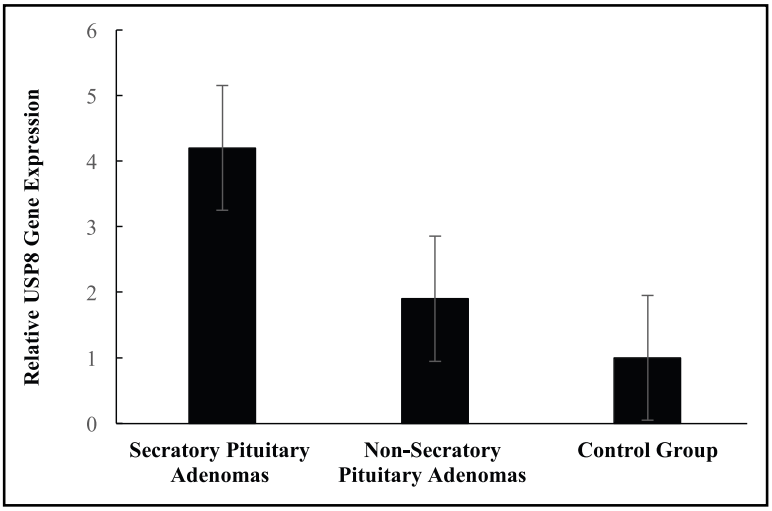


**Fig. 2.** Relative expression levels of USP8 (Ubiquitin-specific protease 8) in secretory, non-secretory pituitary adenomas and, non-adenomatous brain tissue (control group)

prolactin secreting PA: 8.9 [IQR: 2.03-2.15], for adrenocorticotropic hormone (ACTH) secreting PA: 3.4 [IQR: 1.5-16.6], and for growth hormone secreting PA: 2.5 [IQR: 0.6-50.1], $p$=0.6).

Overall, USP8 expression levels did not demonsrate any significant correlation with hormone levels, preoperative adenoma size, Knosp and Hardy classification and, postoperative residual status (Data is not shown here). In corticotropic adenoma group, no correlation between ACTH and USP8 expression levels has been established ($p$=0.3).

## DISCUSSION

Our study revealed that expression levels of the USP8 gene were significantly higher in tissue samples taken from the pituitary adenomas in comparison to the levels in tissue samples taken from the temporal lobe parenchyma. After stratification of pituitary adenomas by their functional status, secretory PA had higher USP8 gene expression levels in comparison to non-adenomatous brain tissue. When further analyzed, there were no statistically significant differences between secretory and non-secretory PA and, between non-secretory PA and non-adenomatous brain tissue. We did not detect a relationship between USP8 expression levels and any feature of corticotroph adenomas.

Ubiquitin is a protein that is expressed in eukaryotic cells and selectively labels protein structures to be degraded (Srinivasan & Nawaz, 2009). Ubiquitination is a posttranslational process that adds ubiquitin and therefore regulates protein degradation, DNA repair, endocytosis and lysosomal functions in the cell (Kulathu & Komander,2012). Deubiquitinases such as USP8 modulate this process by removing ubiquitin from the substrate proteins hence limiting protein degredation (Reyes-Turcu *et al.* 2009). By removing the ubiquitin from EGFR, USP8 prevents degradation of EGFR, resulting in increased EGFR levels. This subsequently promotes cell cycle progression and increased proliferation (Theodoropoulou *et al.* 2015). Furthermore, USP8 expression level has been related to various cancers including but not limited to cholangiocarcinomas, cervical squamous carcinomas and lung carcinomas, in fact recent studies depicted USP8 potential target for resistant non-small cell lung carcinomas (Jing *et al.* 2020, Yan *et al.* 2018, Byun *et al.* 2013). Although gain of function mutations of USP8 have been known to be involved in corticotropinoma tumorigenesis, it is still uncertain whether, without a mutation, USP8 expression levels would contribute to pituitary tumorigenesis as they did in other cancers (Reincke *et al.* 2015, Ma *et al.* 2015).

It has been previously demonstrated that deubiquitination related to USP8 mutation could lead to decreased EGFR downregulation and sustained epithelial growth factor (EGF) activity, hence resulting in increased cell proliferation and enhanced ACTH secretion (Theodoropoulou *et al.* 2015). Since EGF also partakes in pituitary hormone secretion, increased EGF activity secondary to USP8 gain of function mutations also lead to enhanced ACTH secretion (Theodoropoulou *et al.* 2015, Renner *et al.* 1996). In this study USP8 gene expression levels were significant in tissue samples taken from the pituitary adenomas and were reported as 3.7 [IQR: 1.1-50.2] times higher than the CBT levels ($p$=0.002). Moreover, USP8 levels were more prominent in secretory PA. Based on relatively increased USP8 expression in secretory adenomas we propose that USP8 may have an additional role in pituitary hormone secretion. However, the USP8 levels were not different between the tissue samples from the corticotroph adenomas and the samples from other secretory PA. Therefore, our study proposes that USP8 is not limited to corticotroph adenomas in fact, it may play a consequential role in various types of pituitary adenomas regarding pituitary tumorigenesis.

Until now promoters of USP8 gene both in human and animals have not been determined (Giovanna & Martegan, 2013). On the other hand, USP8

mutations have been associated with Cushing's disease. Additionally, it has been proposed that corticotroph adenomas with USP8 mutation secrete greater amounts of ACTH (Sesta *et al.* 20020). No other association of expression or mutations of USP8 with other pituitary hormones or PA have been depicted, yet. However, we could not detect a strong relationship between USP8 levels and ACTH secretion. This may be due to several factors including relatively small sample size and USP8 mutation type. Also, instead of wild type USP8, mutated USP8 might be responsible for increased ACTH secretion.

This study was not without certain limitations. We had relatively small number of non-secretory pituitary adenoma tissues, which may underestimate the role of USP8 function in pituitary hormonogenesis. Additionally, due to ethical issues we could not get samples from normal pituitary tissues, therefore we compared expression levels with non-adenomatous brain tissue, which were taken during nonpituitary surgeries.

In conclusion changes in USP8 gene expression levels may contribute to pituitary tumorigenesis and hormonogenesis. Our findings may shed some light on the pathogenesis of sporadic pituitary adenomas.

## DECLARATIONS

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*Declaration of interest*

The authors declare that they have no conflict of interest.

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