Expression profiles of somatostatin, dopamine, and estrogen receptors in pituitary adenomas determined by means of synthetic multilocus calibrators

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Aims. Pituitary adenomas (PA) are non-invasive benign tumors with a high autopsy prevalence. They are classified according to the type of hormone secreted (prolactin, growth hormone, adrenocorticotropin, thyrotropin, folitropin, or luteinizing hormone). Clinically non-functioning adenomas (CNFA) lacking the typical hypersecretion of hormones make up a significant portion of PA. The aim of the study was to determine the complete expression profiles of somatostatin receptors (SSTR1–SSTR5), dopamine receptors type 2 (D2R), and estrogen receptors (ER1) in various types of PA.
Methods. Adenoma specimens were obtained from 206 patients during transsphenoidal resection. For quantitative analysis, reverse transcription and consequent real-time PCR with synthetic multilocus calibrators (SMC) were used. The obtained data were normalized to the number of transcripts of the beta-glucuronidase gene.
Results. The use of SMC enabled the alignment of individual calibration functions for all the receptors. No relationships between the expression of the receptors and the tumor size, site of extension, gender or age at diagnosis were significant. In growth hormone-secreting adenomas, *D2R* and *SSTR2* transcripts were extensively expressed, followed by *ER1*, *SSTR5*, *SSTR3*, and *SSTR1*. In patients with macroprolactinomas, transsphenoidal resection was indicated because dopamine agonists did not normalize prolactin levels. *D2R*, *ER1* and *SSTR1* transcripts were significantly transcribed. Corticotroph adenomas showed high levels of *D2R* and *ER1* transcripts and lower amounts of *SSTR2* and *SSTR1* transcripts. *SSTR5* transcripts were very low. Subjects with CNFA dominantly expressed *D2R* and *ER1*, followed by *SSTR2* and *SSTR3* mRNA.
Conclusion. We evaluated *SSTR1–SSTR5*, *D2R*, and *ER1* expressions in a large group of pituitary adenomas and we found that determining their individual expression profiles could help when choosing the optimal postoperative treatment.

Key words: pituitary adenoma, somatostatin receptor, dopamine receptor, estrogen receptor, real-time PCR, gene expression

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INTRODUCTION

Pituitary adenomas (PA) are non-invasive benign tumors with a high autopsy prevalence ranging from 10 to 27% (ref.[1]). They are classified according to the type of hormone secreted. The excess of prolactin produced by lactotroph adenomas leads to galactorrhoea, amenorrhoea, infertility, impotence, and hypogonadism. Overproduction of growth hormone (GH) in somatotroph adenomas causes acromegaly or gigantism, while high levels of adrenocorticotropin (ACTH) produced in corticotroph adenomas are associated with Cushing disease and thyrotroph adenomas secreting thyrotropin (TSH) can induce hyperthyreosis. Gonadotroph adenomas release folitropin (FSH) and/or luteinizing hormone (LH). Although clinically non-functioning adenomas (CNFA) are not associated with a typical hypersecretion of hormones, their mass effect results in an optic chiasm as a result of the oculomotor or abducens nerves being compressed, hydrocephalus, and orbital or sinonasal symptoms.

According to the type, location and size of the tumor, therapy of PA includes neurosurgical intervention and/or pharmacological treatment. As tumor recurrence appears in 40–50% of patients after transsphenoidal resection[2], treatment generally involves radiotherapy, somatostatin analogues (SA; i.e. long acting forms of octreotide and lanreotide or pasireotide) and dopamine agonists (DA, i.e., cabergoline, bromocriptine, quinagolide).

Somatostatin receptors (SSTR) are membrane G-proteins that mediate physiological functions of somatostatin in the body[3]. Overexpression of SSTR has been found in PA and many human malignancies[4,5]. SSTR are

divided into five basic types: SSTR1 (*SSTR1* gene location 14q13), SSTR2 (17q24), SSTR3 (22q13.1), SSTR4 (20p11.2), and SSTR5 (16p13.3). Octreotide and lanreotide have a high affinity to SSTR2 and SSTR5, a middle affinity to SSTR3, and low affinity to SSTR1 and SSTR4. Pasireotide possesses an affinity to SSTR1, SSTR3, and SSTR5.

The extensive variability of somatostatin receptor expression in pituitary adenomas has been previously described[6]. Moreover, their expression could be influenced by the number of dopamine receptors type 2 (D2R) and estrogen receptors (ER1) in the membrane of the pituitary cells[7-9].

The aim of the study was to determine the expression profiles of SSTR1–SSTR5, D2R, and ER1 receptors in a large group of pituitary adenomas of various clinical classifications. For quantitative analysis, real-time PCR and synthetic multilocus calibrators (SMC) were used.

MATERIAL AND METHODS

Subjects

The group of patients was made up of 105 men (22–82 years old; median 60) and 101 women (8–87 years old; median 57). Adenoma specimens were obtained from patients during transsphenoidal resection at the Neurosurgery Clinic, Central Military Hospital in Prague and the University Hospital in Hradec Kralove, Czech Republic. The collection and laboratory analysis were conducted with the informed consent of the patients in accordance with the requirements of the Clinical Research Ethics Committees. Diagnoses included 144 CNFA, 44 cases of acromegaly and nine cases of Cushing disease, six macroprolactinomas with low response to dopamine agonists, and three TSH-secreting adenomas. The group of CNFA was split into the following histo-pathological subgroups: gonadotroph adenomas (n = 108), silent ACTH tumors (n=12), plurihormonal tumors (n = 10), *null cell* adenomas (n = 10), and rare cases of clinically silent acromegaly and silent prolactinomas (n = 4).

The sizes of the adenomas ranged from 3 to 50 mm (median 23 mm). We collected 23 microadenomas (<10 mm) and 183 macroadenomas (>10 mm). Eighty-one tumors extended suprasellarly and into the *sinus cavernosus*, 79 extended suprasellarly, 17 intrasellarly, 13 suprasellarly and parasellarly, 8 into the *sinus cavernosus*, 6 suprasellarly, parasellarly and into the *sinus cavernosus*, and 2 parasellarly.

RNA extraction and reverse transcription

A portion of the tumor was used for pathological examination and the remaining part was submerged into a nucleic acid stabilizing solution (RNAlater Tissue Protect, Qiagen, Germany) and frozen at -80 °C until RNA analysis. After tissue homogenization (MagNA Lyser, Roche Diagnostics, Germany), RNA was extracted by Trizol Reagent (Invitrogen, USA) following the manufacturer's instructions. To prevent DNA contamination, the RNA extracts were incubated at 30 °C for 20 min with DNase I Recombinant and Protector RNase Inhibitor (Roche Diagnostics, Germany). DNase I activity was consequently inactivated by adding 0.2M EDTA (pH 8) and heating at 75 °C for 10 min. The RNA concentration and purity were controlled by ultraviolet spectrophotometry using Nanodrop ND-1000 (Thermo Scientific, USA). Following the samples were reverse-transcribed to cDNA with the SuperScript III First-Strand Synthesis (Invitrogen, USA) according to the manufacturer's protocol.

Preparation of synthetic multilocus calibrators

The sequence of the polynucleotide chain (IDT, USA) was composed of eight synthetic segments (Fig. 1) aligned as follows: *D2R* (segment I; 150 bp; NCBI reference sequence code NM_000795.3; positions 1281–1430), *SSTR1* (segment II; 210 bp; NM_001049.2; positions 1891–2100), *SSTR2* (segment III; 140 bp; NM_001050.2; positions 279–418), *SSTR3* (segment IV; 140 bp; NM_001051.4; positions 724–863), *SSTR4* (segment V; 240 bp; NM_001052.2; positions 1–240), *SSTR5* (segment VI; 142 bp; NM_001053.3; positions 1013–1154), *ER1* (segment VII; 307 bp; NM_000125.3; positions 954–1260), and beta-glucuronidase gene, *GUS* (segment VIII; 487 bp; NM_000181.3; positions 1708–2194). The complete chain was inserted into a pUC IDT cloning vector and the constructs were transferred into an *E. coli* competent strain and amplified (IDT, USA). This was followed by purification, spectrophotometric determination of plasmid DNA concentration, and freeze-drying. After reconstituting the freeze-dried DNA molecules in Tris-EDTA buffer, serial dilution (10^9–10^1 copies/μL) in plastic tubes was performed. 20 μL aliquots of the diluted DNA were stored at -80 °C.

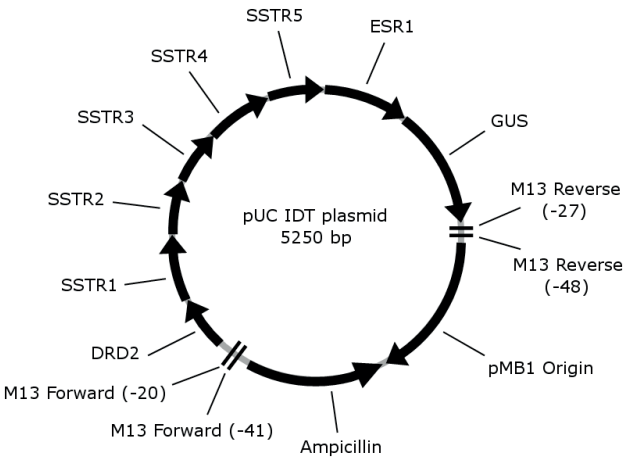

Fig. 1. Alignment of the inserted synthetic sequences in a pUC IDT cloning vector.

Real-time PCR

The master mix (25 μL) contained 12.5 μL TaqMan Universal PCR Master Mix (Applied Biosystems, USA), 5 μL of cDNA, 300 nM of each primer and 200 nM of the hydrolysis fluorescent probe. The sequences of primers and probes for *SSTR1*, *SSTR2*, *SSTR3*, and *SSTR5* summarized in Table 1 have been previously published [10]. *SSTR4* and *ER1* analyses were performed using

Table 1. Sequences of the used primers and probes.

Name	Sequence (5´- 3´)
SSTR1-fw	GCT AGG ACA CTG ACA GCC TTT GA
SSTR1-re	GTA GCC TGA AAG CCT TCC CA
SSTR1-probe	FAM-CCC AAG AAA GGC GCG CGA CAA T-TAMRA
SSTR2-fw	GCC TCC AGG GTC CAT TAA GG
SSTR2-re	ATT GAG TGG CTC ATC CGC C
SSTR2-probe	FAM-AGA ATA AGA TCT CTG GGC TGG CTG GAA-TAMRA
SSTR3-fw	TGG GCC TGC TGG GTA ACT
SSTR3-re	GAT GTA GAC GTT GGT GAC TGA AGG
SSTR3-probe	FAM-CAT CTA TGT GGT CCT GCG GCA CAC G-TAMRA
SSTR5-fw	CTG GTG CCA AGG ACG CT
SSTR5-re	GCT GCC GGA TCC TGT CTG
SSTR5-probe	FAM-ACG CCA CGG AGC CGC GT-TAMRA
D2R-fw	CAA GAC CAT GAG CCG TAG GAA G
D2R-re	TGT GTG TGA TGA AGA AGG GCA G
D2R-probe	FAM-CCC AGC AGA AGG AGA AGA AAG CCA CTC A-TAMRA

Taqman Gene Expression Assays Hs01566620_s1 and Hs00174860_m1 (Invitrogen, USA). The number of *GUS* transcripts was investigated using an *ipsogen* BCR-ABL1 Mbcr (GUS) Kit (Qiagen, Germany).

Quantitative real-time PCR was performed in a RotorGene 6000 thermal cycler (Corbett Research, Australia) under the following conditions: 50 °C 2 min, 95 °C 10 min, then 50 cycles consisting of 95 °C 15 s for denaturation and 60 °C 1 min for both annealing and extension. For the calibration process, diluted SMC aliquots in a range of 10^6–10^1 copies/μL were used. Specimens were tested in duplicate. The obtained data were normalized to the number of *GUS* transcripts and the results are given in arbitrary units.

Statistical analysis

The calculations were carried out by Statistica, version 12 (StatSoft, Czech Republic) and MedCalc, version 5 (MedCalc Software, Belgium). The normality of values was evaluated by the Shapiro-Wilk W test. Data analysis was performed by using the Mann-Whitney U test. Co-expressions of receptors were evaluated by the Spearman rank correlation. *P* values lower than 0.05 were considered statistically significant.

RESULTS

The preparation of SMC enabled us to perform calibration procedures for all the receptors using the same concentration series with no doubts as to their comparability to each other. As demonstrated in Table 2, reaction slopes, efficiencies, and regression coefficients of the receptor assays were very similar. Cycle thresholds (CT) for each calibration point varied in 2–3 cycles.

Table 2. Parameters of calibration functions.

Transcript	Calibration function#				Cycle thresholds (CT) for calibration points DNA copy numbers per reaction				
	Slope	Intercept	r	Efficiency	$5x10^6$	$5x10^5$	$5x10^4$	$5x10^3$	$5x10^2$
SSTR1	-3.42	42.20	0.999	1.96	19.4	22.6	26.1	29.5	33.0
SSTR2	-3.38	42.87	0.980	1.98	20.4	23.6	26.9	30.0	33.8
SSTR3	-3.38	40.05	0.999	1.98	17.6	20.7	23.9	27.3	31.1
SSTR4	-3.31	40.84	0.999	2.00	18.6	21.9	25.3	28.7	32.2
SSTR5	-3.53	42.02	0.999	1.92	18.4	21.9	25.4	28.8	32.5
D2DR	-3.43	42.46	0.999	1.95	19.5	22.8	26.2	29.9	33.2
ER1	-3.46	40.64	0.999	1.94	17.5	20.9	24.2	27.9	31.3
GUS	-3.64	42.51	0.999	1.90	18.1	21.7	25.4	29.0	32.7

#common format of a calibration curve: CT = slope * log(concentration) + intercept; threshold 0.05; r = regression coefficients

SSTR1-*SSTR3*, *D2R*, and *ER1* mRNA were expressed in all 206 examined adenomas. *SSTR4* and *SSTR5* transcripts were detectable in 72% and 81% of them, respectively. The absolute quantity of *GUS* transcripts in the mixtures moved from 1816 to 778305 copies (median 17354 copies). Normalized expressions of the genes ranged from 0.2 to 26796 for *SSTR1*, 0.8–5348 for *SSTR2*, 0.1–1041 for *SSTR3*, 0–54 for *SSTR4*, 0–1395 for *SSTR5*, 0.2–17356 for *D2R* and 0.1–14359 for *ER1*. No significant differences in expression levels between men and women were observed. Also, no dependence of the expressions on the age at diagnosis was found.

The numbers of *D2R*, *ER1*, and *SSTR1* transcripts were highest in the subjects with Cushing disease and prolactinomas (Mann-Whitney U test, $P<0.001$, Table 3). *SSTR2* expression was significantly higher in acromegaly and TSH-secreting adenomas than in the others ($P<0.001$). *SSTR3*, *SSTR4*, and *SSTR5* expressions in PA were lower in general. Significantly more *SSTR3* transcripts were found in CNFA and acromegaly than in other PA types ($P=0.002$ and P=0.008, respectively), while relatively higher levels of *SSTR5* were found in patients with acromegaly and prolactinomas.

Table 3. Normalized expression of receptors (median values) in pituitary adenomas.

	GH (n=44)	PRL (n=6)	CORTI (n=9)	CNFA (n=144)	TSH (n=3)
SSTR1	12	130	46	7	11
SSTR2	174	18	49	25	551
SSTR3	14	1	7	23	0
SSTR4	0	0	1	0	1
SSTR5	17	17	2	0	0
D2R	516	3172	1099	325	440
ER1	32	1665	607	54	68

GH growth hormone secreting adenomas, PRL prolactinomas, CORTI corticotroph adenomas, CNFA clinically non-functioning adenomas, TSH thyrotropin secreting adenomas

The median size of CNFA was 24 mm (in a range of 10–50 mm, except for one 7 mm adenoma associated with cephalea and hemianopsia), 16 mm in GH-secreting adenomas (3–40 mm), 8 mm in corticotroph adenomas (4–30 mm), 24 mm in prolactinomas (9–56 mm), and 24 mm in thyrotroph adenomas (5–30 mm). No relationships between the expression and the tumor size or sites of the extension were significant.

When evaluating co-expressions of the receptors, positive Spearman rank correlations between *ER1* and *D2R* (r=0.51; $P<0.001$; Fig. 2, left part), and between *SSTR2* and *D2R* (r = 0.41; $P < 0.001$; Fig. 2, right part) in PA were observed. Other relationships among the receptors were weak or missing. With the exception of *ER1* and *D2R* (r = 0.69, $P < 0.001$), we found no significant co-expressions of the receptors in the group of CNFA group.

DISCUSSION

Laboratory testing of SSTR or other receptors is traditionally performed via autoradiography and immunohistochemistry[8,11-13]. Results obtained by these methods are, however, influenced by the variety of usable epitopes, by the heterogeneous dimerization and distribution of receptors, and by their internalization and diffusion into the cytosol and/or endosomes[14]. Therefore, no correlations between the results of immunohistochemical analysis and the number of RNA transcripts in pituitary adenomas have been found[15]. Despite providing no information about the tissue morphology, real-time PCR could provide more precise quantitative data on *SSTR*, *D2R*, and *ER1* expression profiles if the proper calibration process is used and if normalization to a house-keeping gene is carried out.

Calibration standards are commonly prepared by inserting the appropriate DNA or RNA fragments into individual vectors and performing a series of molecular cloning procedures. The spectrophotometric analysis of the amounts of fragments in the standards depends on the imprecision of the method impairing the homogeneity of calibrations and on the reliability of the data obtained.

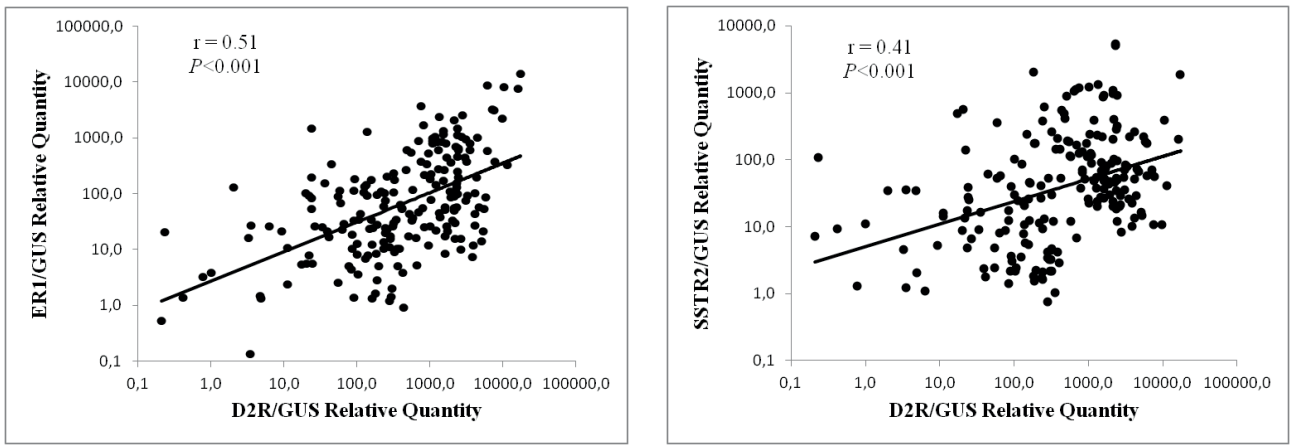

Fig. 2. Co-expressions of *ER1* and *D2R* (left part) and *SSTR2* and *D2R* (right part) genes in PA drawn in the logarithmic scale.

Our newly developed calibration method uses gene synthesis technology along with the insertion of several DNA fragments into a single cloning vector. This approach ensures equivalent copy numbers of *SSTR1-5*, *D2R*, *ER1*, and *GUS* fragments in the prepared calibration standards.

For normalization we chose the *GUS* house-keeping gene. Compared with other reference genes (*G3PDH*, beta-actin, *HPRT*, cyclophilin) (ref.[6,7]), a lower expression of the *GUS* gene in the pituitary tissue enables the *SSTR*, *D2R*, and *ER1* values to be distributed on a wider scale, from units to hundreds of thousands of arbitrary copy numbers.

Cytoplasmatic SSTR mediate physiological functions of somatostatin in the body, including modulation of endocrine and exocrine secretion, inhibition of cell proliferation (SSTR1, SSTR2, and SSTR5), and initiation of apoptosis (SSTR3 and partly SSTR2) (ref.[12,16]). In our group of 44 subjects suffering from acromegaly, *D2R* and *SSTR2* transcripts were extensively expressed, with *ER1*, *SSTR5*, *SSTR3*, and *SSTR1* lesser so expressed. Similar results focusing exclusively on SSTR receptors have been published by others[6,17]. The same profile also showed the three patients with rare TSH-secreting PA.

Octreotide and lanreotide are somatostatin analogues with a specific affinity to SSTR2 and SSTR5. If used in acromegaly treatment (transsphenoidal resection is the primary treatment), they normalize GH levels in up to 70% cases of GH-secreting PA (ref.[18]); SA-resistant acromegaly expresses lower amounts of *SSTR2* mRNA (ref.[19]). Finding high levels of *D2R* in these subjects leads to the consideration of DA as a means for overcoming SA-resistance[20].

Prolactinomas are primarily treated with DA that enable the normalization of elevated prolactin levels in 90% of cases involving microprolactinomas and 70% of macroprolactinomas[16]. In our series of macroprolactinomas, transsphenoidal resection was indicated because DA did not normalize prolactin levels. Despite this, we observed the highest levels of *D2R* in these tumors out of all those tested. Also, we found in them a high expression of *ER1* and *SSTR1* genes. The associations between *ER1* and *SSTR* co-expressions have been previously described in CNFA[7]. Since estrogen hormones induce the transcription of *SSTR2* and *SSTR3* genes[8,21], the administration of ER1 modulators should probably increase the chance of inhibiting prolactin synthesis in DA-resistant prolactinomas. In such a therapeutic approach, the expression profile (*SSTR*, *D2R*, and *ER1*) and serum prolactin concentrations should be measured[22].

The first line of treatment of ACTH-secreting adenomas is transsphenoidal resection. The data published so far has shown a dominant expression of *SSTR5* and *D2R* and lower levels of *SSTR2* transcripts in this type of PA (ref.[23]). Therefore, preoperative reduction of ACTH and consequent reduction of cortisol levels in the body could be reached by SA (pasireotide) or DA (ref.[24,25]). In our study we investigated nine corticotroph adenomas. In their expression profiles, high levels of *D2R* and *ER1* dominated; *SSTR2* and *SSTR1* transcripts were significantly lower in them. *SSTR5* copy numbers were very low, in three subjects even under the assay detection limit. Considering the significant elevation of D2R receptors in these tumors, therapy by cabergoline should be a more effective postoperative approach, although it would not be effective in the long term[26].

Most subjects in the study were diagnosed as CNFA with a high expression of *D2R* and *ER1*, followed by *SSTR2* and *SSTR3*. The amounts of *SSTR1*, *SSTR4*, and *SSTR5* transcripts were significantly lower, as reported earlier[5,7]. Moreover, CNFA are primarily treated by transsphenoidal resection. Relapses after complete extraction appear in 13% of individuals. In the case of postoperative residuum, the risk of a relapse rises to 41% (ref.[27]). Early postoperative radiotherapy decreased tumor recurrence. If *D2R* expression in PA is sufficient, the DA postoperative treatment could be considered. Unfortunately, our data showed that *D2R* expression in CNFA is lower than in prolactinomas, which would thus limit wider usage of DA in the postoperative treatment and primary pharmacological treatment of CNFA.

CONCLUSION

In conclusion, we have evaluated complete *SSTR1-5*, *D2R*, and *ER1* expression profiles in the largest group of resected pituitary tumors (to our knowledge) ever collected. Since the therapeutic effect of the mentioned hormone analogues, agonists, and modulators depends on the quantity and functionality of the appropriate receptors, determining their individual expression profiles could prove beneficial when choosing the optimal postoperative treatment for pituitary adenomas.

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Conflict of interest statement: None declared.

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