

Dopamine 2 and somatostatin 1–5 receptors coexpression in clinically non-functioning pituitary adenomas.

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Short title: Receptor coexpression in CNFAs

Summary

Purpose: This study investigated quantitated expression of dopamine 2 receptor (D2R) and somatostatin receptors of the five types (SSTR1–SSTR5) in a large series of clinically non-functioning pituitary adenomas (CNFAs). Co-expression of these receptors in individual adenomas was studied as well as correlation between receptor types.

Patients and methods: Adenoma tissue from 198 patients who underwent surgery for CNFAs was analyzed by immunohistochemistry and quantitative real-time PCR.

Results: *D2R* and *SSTR1–3* mRNA was expressed in all 198 adenomas. *SSTR4* and *SSTR5* were detectable in 85% and 61% of adenomas, respectively. Expression of D2R was significantly higher than that of the somatostatin receptors. The median relative expressions were as follows from highest D2R >> SSTR3 > SSTR2 > SSTR1 > SSTR5 > SSTR4. High relative expression (ratio to β -glucuronidase mRNA > 1) of D2R was found in 60% of tumors, high expression of SSTR1 in 7.5%, SSTR2 in 7%, SSTR3 in 4% and SSTR5 in 0.5%. The quantity of D2R correlated positively with expression of SSTR2 and SSTR3, and negatively with SSTR1 and SSTR5. Among histological adenoma types, *SSTR1* was significantly higher in null-cell adenomas and *SSTR3* was lower in silent corticotroph adenomas.

Conclusions: In CNFAs, high expression of somatostatin receptors is much less common than that of D2R, and co-expression of both these receptors is exceptional. D2R and SSTR3 seem to be the most promising targets for pharmacological treatment.

Introduction

Clinically non-functioning pituitary adenomas (CNFAs) represent one third of human pituitary tumors. An estimated prevalence of all pituitary tumors is 16.7% (Ezzat et al. 2004).

Diagnosis of CNFAs is often determined when the tumor had grown to the stage of macroadenoma and presents with signs of mass effect. The majority of CNFAs produce either low, non-significant amounts of hormones or defective hormones. No more than 10% of CNFAs are immunohistochemically negative; these are called null-cell adenomas (Jaffe 2006).

Transsphenoidal surgery is the primary option for CNFAs requiring treatment. However, because of frequent supra- or parasellar extension, surgery is infrequently curative, leaving tumor remnants that regrow in 12–58% of patients within 5 years of follow-up (Brochier et al. 2010, Greenman et al. 2003). The only independent predictors for tumor regrowth are: a) preoperative presence of cavernous sinus invasion, and b) postoperative suprasellar extent of residual tumor (Greenman et al. 2003). In postoperative management of patients, there is no clear strategy. We can choose between reoperation, Leksell-gamma knife surgery or pharmacological treatment. Each option has its drawbacks. The presence of somatostatin (SSTR) and dopamine 2 subtype (D2R) receptors in CNFAs has been proved by several methods including radioligands, *in-situ* hybridization, RT-PCR, and quantitative real-time RT-PCR. However, empirical use of dopamine agonists (DA) or somatostatin analogues (SA) alone or in combination has been effective in only a few cases (Colao et al. 2008). Recently, new chimeric compounds “dopastatins” showed promising efficacy *in vitro* (Florio et al. 2008). In contrast, pharmacological treatment of CNFAs has not been successful. Highly variable expression or homo/heterodimerization of receptors in the pituitary gland partly could explain this discrepancy (Neto et al. 2009, Renner et al. 1998, Taboada et al. 2007). Thus, knowledge of receptor mRNA expression patterns would help in the targeted therapy

(Babu et al. 2014). The aim of this study was to quantify coexpression of SSTR1-5 and D2R in a large series of CNFAs and to correlate the results with the pathological classification of adenomas.

Patients and methods

Clinically non-functioning adenomas were diagnosed based on the absence of hypersecretory laboratory markers. Specifically, the IGF-I level was normal or low for the particular age, and the serum prolactin level was normal or only slightly elevated to a level considered appropriate to stalk compression seen on MRI. Urinary free cortisol was not elevated. Serum free thyroid hormones were normal or lower. Luteinizing hormone, Follicle-stimulating hormone and testosterone, free testosterone (or estradiol in women) were evaluated. Diagnosis of adenoma was confirmed by pathological investigation after surgery. We enrolled 198 patients who fulfilled study criteria. Several patients from our previous analysis of D2R expression (Gabalec et al. 2012) from whom we had cDNA stored for further analysis were also included. The study was approved by the Ethics Committee; patients had all signed informed consent forms. Subjects were 113 men and 85 women, aged 15–87 years (mean \pm standard deviation 60.4 ± 13.1). All tumors except of one were macroadenomas; sized (at their largest diameters) 7–53 mm (mean \pm standard deviation 25.9 ± 8.8 mm; median: 25 mm). The most common indications for operation were bitemporal hemianopsia or visual defect in 89 cases (45%), diplopia due to nerve palsy in 26 cases (13 %), progression of tumor growth on MRI in 18 (9%) and headache in 21 (11%), or in 44 (22%) in combination. Most patients underwent surgery by transsphenoidal endoscopic approach. Three patients needed subfrontal surgery due to the adenoma extent. At initial evaluation, hypopituitarism was present in 50 patients, diabetes insipidus in 4 cases, 26 patients had hypogonadism and only 2 subjects with hypogonadism had substitution prior to diagnosis.

Pathology

Tissue specimens were investigated as described in our previous work. (Gabalec et al. 2012, Cap et al. 2003) Briefly, adenoma tissue specimens obtained at surgery were immediately fixed in 10% formalin, routinely processed, and subsequently embedded in paraffin. Sections 5 µm thick were stained with hematoxylin–eosin and Goldberg’s trichrome. The Gömöri silver impregnation of reticuline was used to enhance staining of fibrous tissue. In total, 133 specimens from 75 men and 58 women, aged 23–87 years (61.4 ± 12.2 years) underwent immunohistochemical analysis. For immunohistochemistry (IHC), serial sections from the same paraffin blocks were usually exposed to a heat-mediated antigen retrieval procedure in citrate buffer, pH 6.0, using a microwave oven. We investigated the following pituitary hormones: ACTH (monoclonal mouse antibodies, Dako, Denmark); GH; prolactin; and specific β -subunits of the dimeric glycoproteins TSH, FSH, LH, and their common α -subunit (monoclonal antibodies, Immunotech, Prague, Czech Republic). We used the avidin–biotin technique and diaminobenzidine staining. Expression of each hormone was described semiquantitatively in 4 stages: 0: absolutely negative; 1: slight positivity in < 15% of cells; 2: positivity in 15–70% of cells; and 3: strong positivity in > 70% of cells.

Quantitative real-time RT-PCR

Details of RNA extraction, reverse transcription and quantification were described previously. (Gabalec et al. 2012) Briefly, for RT-PCR analysis, small tissue samples were immediately stored in RNAlater (Qiagen, Hilden, Germany) according to the manufacturer’s instructions and frozen at -80° C for RNA stabilization. RNA was isolated by Trizol Reagent (Invitrogen, USA) following manufacturer’s instructions and transcribed to cDNA by SuperScript III First-Strand Synthesis (Invitrogen, USA). We made a real-time PCR master mix of 5 µl of cDNA,

12.5 µl TaqMan Universal PCR Master Mix (Life Technologies, USA), 300 nM of each primer and 200 nM of hydrolytic fluorescent probe. The probe and primers for D2R, SSTR1, 2, 3 and 5 were published previously (O'Toole et al. 2006). SSTR4 analyses were performed using Taqman Gene Expression Assays Hs01566620_s1 (Life Technologies, USA). After incubation for 2 min at 50°C and 10 min denaturation at 95°C, we performed 50 PCR cycles of 15 s denaturation at 95 °C and 60 s annealing and elongation at 60 °C, on a Rotor-Gene 6000 (Corbett, Australia). Serial diluted plasmids pCR4 (Invitrogen, USA) with SSTR1-5 inserts (Generi Biotech, Czech Republic) were used for calibration. Taking into account RNA degradation processes, mRNA quantities were normalized to that of β-glucuronidase (*GUS*) housekeeping gene (Qiagen, Germany).

Statistics

mRNA quantities were expressed both absolutely, in copies per 5 µl of cDNA, and relatively as the ratio of the receptor subtype to housekeeping gene copies. The distribution of values in the whole sample and by groups according to the immunocytochemical classification proved to be non-Gaussian using Kolmogorov–Smirnov test (with Lilliefors' correction). For this reason, the groups were characterized by median and percentiles and the Mann–Whitney rank sum test was used to evaluate differences between individual groups. Comparisons were also done with the use of Kruskal–Wallis analysis of variance on ranks (Dunn's method).

Correlation between various receptors expression was evaluated using Spearman Rank Order Correlation. $P < 0.05$ was considered significant. SigmaStat version 3.5 software (Systat Software, San Jose, USA) was used.

Results

qRT-PCR analysis

D2R and *SSTR1-3* mRNA was expressed in all 198 adenomas examined. *SSTR4* and *SSTR5* were detectable in 85% and 61% of adenomas, respectively. Absolute median values of mRNA expression were 1380 copies/ μ l for *SSTR1* (range: 24–1144017), 4701 copies/ μ l for *SSTR2* (5–577816), 5911 copies/ μ l for *SSTR3* (1–112093), 2 copies/ μ l for *SSTR4* (0–2660), 14 copies/ μ l for *SSTR5* (0–59631) and 222075 copies/ μ l for *D2R* (1–2580406). After normalization to the *GUS* housekeeping gene the median of relative mRNA expression was 2.34 for *SSTR1*, 8.26 for *SSTR2*, 11.38 for *SSTR3*, 0.01 for *SSTR4*, 0.02 for *SSTR5* and 212.20 for *D2R*. Expression of *D2R* was significantly higher than that of the somatostatin receptors. Median relative expressions were as follows from highest *D2R* >> *SSTR3* > *SSTR2* > *SSTR1* > *SSTR5* > *SSTR4* (Fig. 1).

Expression of *D2R* correlated positively (Correlation Coefficient 0.199, P 0.01) with adenoma size. No correlation with size was found for other receptors. Receptor expression was not influenced by tumor extension.

Expression of *D2R* correlated positively with expression of *SSTR2* and *SSTR3* and inversely with expression of *SSTR1* and *SSTR5* (Fig. 2, Table 1). *D2R* expression was high (relative expression > 1) in most CNFAs, whereas expression of individual somatostatin receptor types was much lower, high expression being an exception. (Fig.3)

High expression of somatostatin receptors was rare (15/198 for *SSTR1*, 14/198 for *SSTR2*, 8/198 for *SSTR3* and 1/198 for *SSTR5*, respectively; Table 2). Coexpression of both *SSTR* and *D2R* in high quantities was even less frequent.

qRT-PCR analysis of adenomas according to immunohistochemistry

Among 133 immunohistochemically analyzed specimens, we saw 9 (6.8%) null-cell adenomas, 103 (77.4%) gonadotrophs, 11 (8.3%) silent corticotroph tumors and 10 (7.5%) plurihormonal tumors. Receptors mRNA median relative expression for different subtypes is shown in Table 3.

The median of relative mRNA expression showed the same pattern as the complete group. In contrast, *SSTR3* and *D2R* expressions were significantly lower in silent ACTH adenomas. (Kruskal–Wallis analysis on ranks, $P < 0.001$ and $P < 0.01$, respectively). *SSTR1* was expressed significantly more in null cell adenomas than in gonadotrophs (Kruskal–Wallis analysis on ranks, $P < 0.021$). Expression of *SSTR1* was also higher in null cell adenomas than other subtypes, but this was not statistically significant. (Fig. 4)

Discussion

Transsphenoidal surgery is a standard approach to treating CNFAs. However, management of postoperative residual tumor is controversial. Dopamine agonists are a possible treatment option, as CNFAs express dopamine receptors. Greenman et al. treated 33 patients with residual tumor after surgery. Tumor remnants volumes decreased in 11 cases after DA treatment, but *D2R* expression was not determined (Greenman et al. 2005). Pivonello et al. proved that tumor shrinkage after DA treatment correlates with *D2R* expression (Pivonello et al. 2004). Results of treatment with DA in CNFAs were reviewed by Colao (Colao et al. 2008). Cumulative evidence for tumor shrinkage in CNFAs after treatment with DA was 27.6%. This is in agreement with the finding that about 20–25% CNFAs express *D2R* in relatively high quantities (Su et al. 2012). High variability in dopamine 2 receptor expression (in agreement with other studies (Neto et al. 2009) also concurs with our work (Gabalec et al. 2012).

SSTR1, SSTR2, SSTR3 and SSTR5 are expressed in pituitary adenomas and in normal pituitary tissue. In comparison, SSTR4 expression is low or missing (Neto et al. 2009, Taboada et al. 2007). The effect of somatostatin and its analogues is determined by the binding receptor. Each receptor starts different signal pathways and intracellular response (Hofland et al. 2003, Ferone et al. 2009, Zatelli et al. 2004). Treatment with somatostatin analogues in CNFAs has poor results. Data with short-acting octreotide showed tumor volume reduction only in 12%, whereas most tumors remained stable (Colao et al. 2008). A study with octreotide LAR in CNFAs *in vivo* showed stable tumor remnants in the majority of cases and tumor volume enlargement in 19% of cases (Fusco et al. 2012). Conversely, *in vitro* studies with somatostatin and lanreotide showed significantly inhibited cell proliferation in CNFAs (Florio et al. 1999). In another study SA binding SSTR1, SSTR2 and/or SSTR5 inhibited cell viability and alpha-subunit secretion *in vitro* (Zatelli et al. 2004). In CNFAs, our work supports the findings of Taboada et al. that SSTR3 has the highest expression levels followed by SSTR2. Other authors report predominant and highly variable expression of SSTR2 and SSTR3 (Babu et al. 2014, Florio et al. 2008, Saveanu et al. 2001, Saveanu et al. 2009, Tateno et al. 2009), but SSTR5 was not reported in these papers or had low expression. We observed SSTR5 expression in 61% of cases and SSTR1 expression in all cases, which is more than was reported by Taboada (Taboada et al. 2007). However, Babu reported SSTR5 expression in 82% of patients (Babu et al. 2014). High variability of expression together with preferential binding of current analogues to SSTR2 could explain the low efficacy of SA. To date, no studies that use qRT-PCR correlate the number of receptor copies in CNFAs with response to SA.

Coexpression of dopamine and somatostatin receptors was evaluated only in few studies. Coexpression of SSTR2, SSTR3 and D2R in most evaluated CNFAs had highly variable

expression levels (Neto et al. 2009, Florio et al. 2008, Saveanu et al. 2009, Nishioka et al. 2011). In all series somatostatin receptors are expressed much less than D2 receptors. (Neto et al. 2009, Florio et al. 2008, Saveanu et al. 2009). In our study D2R expression was also higher by one order than SSTR3 or SSTR2 expression. Similar data were reported by Saveanu for 58 CNFAs (Saveanu et al. 2009). The same patterns of D2R and SSTR expression were also seen in CNFAs cell cultures (Florio et al. 2008). High numbers of copies in both somatostatin and dopamine receptors were seen in 3 of 17 patients in the study of Babu (Babu et al. 2014). Evidence indicates that D2R and SSTR can form heterodimers that influence their binding capacity, signalization and internalization. After heterodimerization of SSTR5 with D2R or SSTR2 with D2R, new receptors presented with new conformation, higher activities and binding capacities for both SA and DA (Baragli et al. 2007, Rocheville et al. 2000). Adenomas with high expression of somatostatin receptors are rare and those with high coexpression of both somatostatin and dopamine receptors are an exception. Therefore chimeric compound BIM-23A760 that bind both somatostatin and dopamine receptors did not inhibit cell cultures more than DA alone (Florio et al. 2008, Gruszka et al. 2006).

We found negative correlation between D2R expression and expression of SSTR1 and SSTR5, but D2R expression correlated positively with expression of SSTR2 and SSTR3. This is an original finding, different from that of Neto et al. who found no correlation in 14 CNFAs (Neto et al. 2009). The number of samples probably explains the discrepancy, as the relationship is not very close. Some adenomas had low D2R expression and high somatostatin receptor expression, especially of the 1 and 5 subtypes.

With regard to pathological subtypes of CNFAs, we found significantly lower expression of SSTR3 in silent corticotrophs. The median relative SSTR5 expression was higher than in other subtypes, but this difference was not significant. Higher SSTR5 expression was

previously reported in corticotroph adenomas (de Bruin et al. 2009). Tateno also reported high SSTR5 expression in corticotroph adenoma, but did not observe significant differences between silent corticotrophs and other CNFAs. SSTR5 expression was even lower than in CNFAs (Tateno et al. 2009). Again, this discrepancy might be due to the number of examined adenomas.

To our knowledge no previous study has correlated D2R and SSTR coexpression to pathological subtype. Compared with gonadotroph and null-cell adenomas, where D2R expression was predominant and significantly higher than SSTR, SSTR3 and SSTR2 were the most expressed receptors (although not significantly) in plurihormonal adenomas and silent corticotrophs, respectively. SSTR1 was statistically more expressed in null-cell adenomas than in other subtypes. Evaluation of other subtypes is limited by small number of cases other than gonadotrophs.

To conclude, in CNFAs, high expression of somatostatin receptors is much less common than that of D2R, and coexpression of both these receptors is exceptional.

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Figure legends

Fig. 1 Relative expression of SSTR1-5 and D2R normalized to GUS in CNFAs. Upper border of the box: 75th percentile; lower border: 25th percentile; line: median; whiskers: 99th percentile range; small square: arithmetic mean; crosses: extreme values.

Fig 2 Correlations of dopamine receptor (D2R) and four somatostatin receptor types relative expression. Statistical significance was established by Spearman Rank Order Correlation.

Fig. 3: Number of adenomas with high (>1), medium (0.1–1) and low (<0.1) relative expression of individual receptors, normalized to percentage of GUS.

Fig. 4 Relative expression of SSTR1R normalized to GUS in individual CNFA pathology types. Null-cell adenomas and gonadotrophs differed significantly (* $P = 0.021$).

Tables

Table 1. Correlation coefficients and their significance by Spearman Rank Order Correlation

	SSTR2	SSTR3	SSTR4	SSTR5	D2R
SSTR1	0.384 p>0.001	0.0393 p=0.583	0.394 (p>0.001)	0.304 (p>0.001)	-0.273 (p>0.001)
SSTR2		0.335 (p>0.001)	0.0283 p=0.755	0.0245 p=0.732	0.221 p=0.002
SSTR3			0.026	-0.0159	0.315

	p=0.775	p=0.825	(p>0.001)
SSTR4		0.289	-0.0726
		p=0.001	p=0.424
SSTR5			-0.266
			(p>0.001)

Table 2. Number of patients with high somatostatin receptors (relative mRNA expression/GUS > 1), relative to D2R expression. (n= 198 adenomas)

	SSTR1	SSTR2	SSTR3	SSTR5
D2R/GUS > 1	4	8	5	0
D2R/GUS < 1	11	6	3	1
Total	15	14	8	1

Table 3. Median *SSTR1-5* and *D2R* mRNA expression (relative to *GUS*) for different adenoma subtypes.

Median relative mRNA expression/*GUS*

	SSTR1	SSTR2	SSTR3	SSTR4	SSTR5	D2R
Null cell adenomas	34.00	12.20	0.50	0.01	0.00	134.03
Gonadotrophs	2.18	6.55	11.09	0.00	0.02	109.10
Silent ACTH	2.77	4.53	0.99	0.00	1.90	2.01
Plurihormonal	4.35	3.81	24.06	0.00	0.00	13.70

Figures

Fig. 1 Relative expression of SSTR1-5 and D2R normalized to GUS in CNFAs. Upper border of the box: 75th percentile; lower border: 25th percentile; line: median; whiskers: 99th percentile range; small square: arithmetic mean; crosses: extreme values.

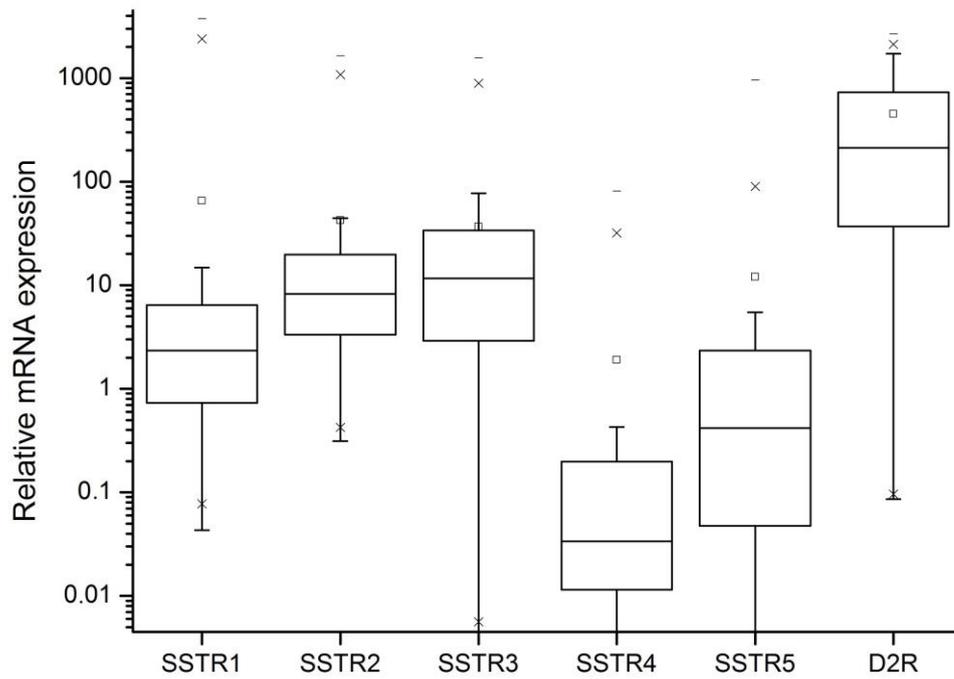


Fig 2 Correlations of dopamine receptor (D2R) and four somatostatin receptor types relative expression. Statistical significance was established by Spearman Rank Order Correlation.

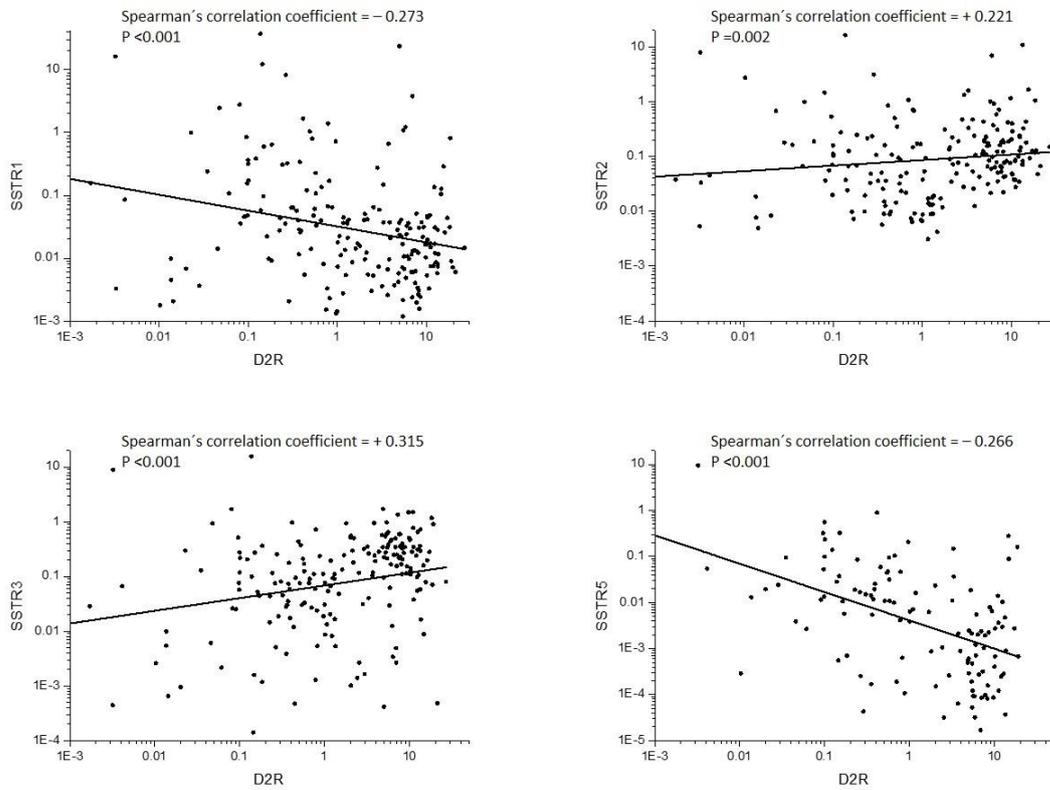


Fig. 3: Number of adenomas with high (>1), medium (0.1–1) and low (<0.1) relative expression of individual receptors, normalized to percentage of GUS.

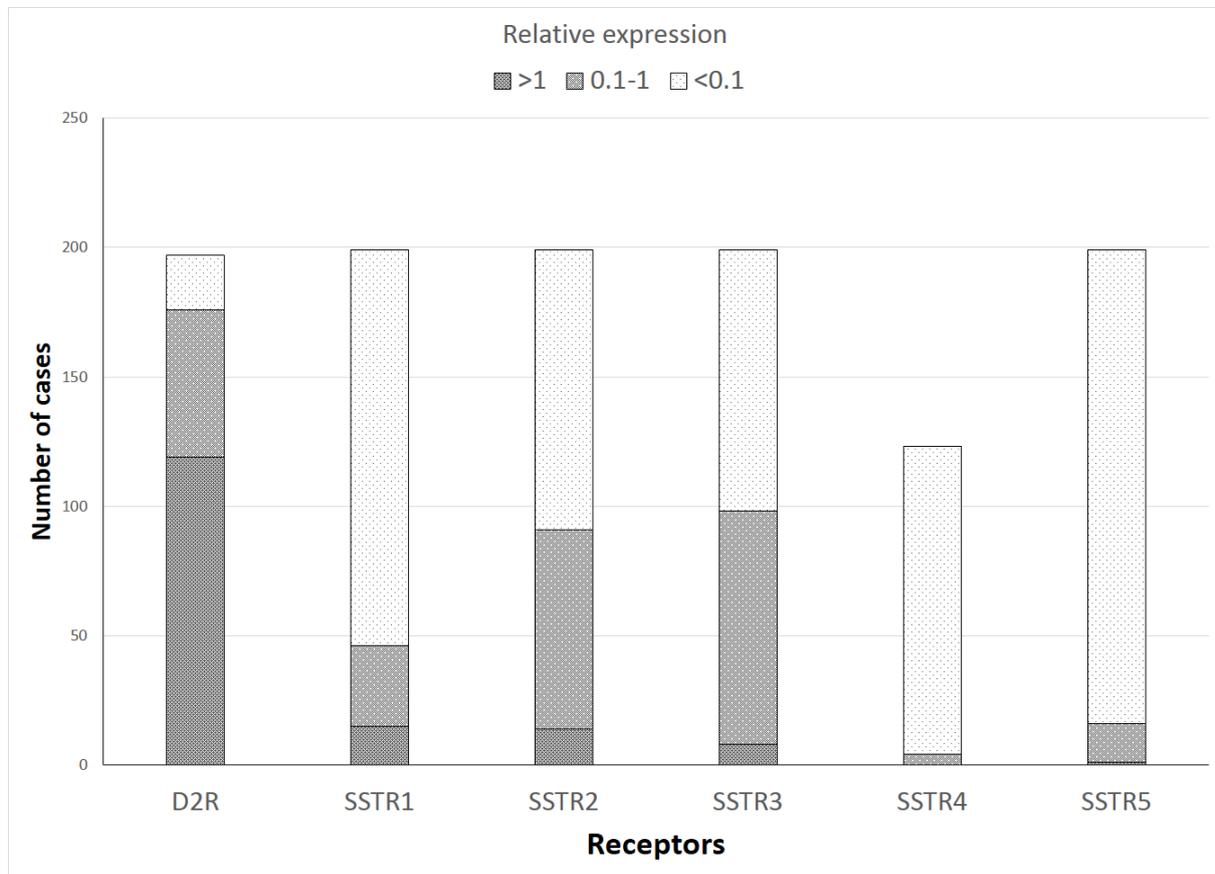


Fig. 4 Relative expression of SSTR1R normalized to GUS in individual CNFA pathology types. Null-cell adenomas and gonadotrophs differed significantly (* $P = 0.021$).

