Immunohistochemical localization and functional characterization of somatostatin receptor subtypes in a CRH-secreting adrenal pheochromocytoma: review of the literature and report of a case

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Somastostatin receptors are frequently expressed in pheochromocytoma, but data on somatostatin receptor subtyping are scanty and the functional response to the somatostatin analogue octretide is still debated. We report an unusual case of pheochromocytoma, causing ectopic Cushing’s syndrome due to CRH production by the tumour cells, in a 50-yr-old woman. Abdominal computed tomography revealed an inhomogeneous, 9-cm mass in the right adrenal gland, and [¹¹¹In-DTPA] octreotide scintigraphy showed an abnormal uptake of the radiotracer in the right perirenal region, corresponding to the adrenal mass. The patient underwent laparoscopic surgery and formalin-fixed and paraffin-embedded samples were studied. The tumour was extensively characterized by immunohistochemistry and somatostatin receptor (SSTR) subtypes expression was analyzed. Histological and immunohistochemical examination of the surgical specimens displayed a typical pheochromocytoma, which was found to be immunoreactive to S-100, chromogranin A and neurofilaments. Immunostaining for SSTR subtypes showed a positive reaction for SSTR1, SSTR2A, SSTR2B, SSTR3, SSTR5, SSTR6 antisera on tumour cells. The intense and diffuse immunostaining for Corticotropin Releasing Hormone (CRH) antiserum indicated that Cushing’s disease was dependent on CRH overproduction by the pheochromocytoma, in which no immunostaining for adrenocorticotropic hormone was found. Our report confirms the heterogeneity of the pattern of SSTR expression in pheochromocytomas, and provides further evidence for functional SSTR subtype SSTR2 in a subgroup of pheochromocytomas, suggesting that these tumours may represent potential target for octreotide treatment.

Key words: pheochromocytoma, neuroendocrine tumours, somatostatin receptors, octreotide, CRH.

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Patient and Methods

Patient

A 50-yr-old woman referred to our Endocrine Unit for help in the management of severe hypokalaemia and hypertension. At the time of...
presentation, she displayed typical Cushing’s features. Plasma ACTH and serum cortisol levels were elevated (73 pmol/L, n.v. 2-11, and 3773 nmol/L, n.v. 215-1000, respectively), without circadian rhythm (midnight cortisol 2905 nmol/L). Mean 24-h urinary free cortisol excretion (UFC) was markedly elevated (1627 nmol/24h; n.v. 206-744), while plasma and urinary epinephrine, normetanephrine and metanephrine concentrations were within the normal range. Serum Chromogranin A levels were markedly increased (1060 ng/mL; n.v. <100). Abdominal computed tomography (CT) demonstrated a 9 cm round mass with calcifications and central necrosis in the right adrenal gland. No other abnormalities were detected either by CT of neck and chest or by magnetic resonance imaging of the pituitary. A whole-body scintigraphy (WBS) with [111In-DTPA] octreotide (Octreoscan®) showed abnormal uptake in the area corresponding to the adrenal mass demonstrated by CT (Figure 1), indicating expression of the SSTRs by the tumour cells. Thus, medical treatment with the somatostatin analog octreotide (0.1 mg s.c. two times daily) was started. This treatment induced a dramatic improvement in clinical signs and symptoms, which was accompanied by rapid normalization of plasma ACTH, serum cortisol and UFC levels, serum potassium and glucose levels. When her clinical condition improved, the patient underwent surgery. The post-operative course was uncomplicated, and hydrocortisone replacement therapy was not necessary. Clinical features of Cushing’s syndrome gradually disappeared of all and at the last follow-up, three years after surgery, the patient is free of disease.

**Histological and immunohistochemical evaluation**

The neoplastic surgical sample was fixed in 10% neutral formalin for 24 hours at room temperature; successively, some representative specimens were embedded in paraffin at 55°C and cut into 5 µm thick consecutive sections to perform Haematoxylin-Eosin (H&E) routine histological stain and immunohistochemical procedures. The local Ethics Committee approved the experimental protocol and an informed consent was obtained from the patient.

The immunohistochemical characterization of the tumour was performed utilizing, separately, the following antisera: a) monoclonal antisera against chromogranin A (working dilution 1:100), neurofilaments (wd 1:100), human Ki-67 antigen (clone MIB-1, wd 1:200) and ACTH (wd 1: 75), (all from DAKO, Carpinteria, CA, USA); b) polyclonal antisera against S-100 (DAKO, wd 1:300), Corticotropin Releasing Hormone (CRH), and Pro-opiomelanocortin Precursor (POMC) (all from Abcam, Cambridge, UK, wd 1:100).

Antigen retrieval procedures (microwave oven exposure for 3 cycles x 5 min at 100°C, in 10 mM citrate buffer, pH 6.0) were carried out for the antisera against ACTH, chromogranin A and human Ki-67 antigen. A pre-treatment with pronase (DAKO) 0.05% in phosphate-buffered saline (PBS) for 5 min at room temperature was applied before the immunostaining for S-100. The immunostaining of all antisera, either monoclonal or polyclonal, was preceded by steps with 0.1% H₂O₂ in methanol to block the intrinsic peroxidase activity and with normal sheep serum to prevent aspecific adherence of serum proteins; the incubation was carried out at 4°C for 16 hrs in a moist chamber. Finally, the biotin-streptavidin-peroxidase method (LSAB2 System-HRP multilink universal kit from DAKO) was applied. To develop the reaction, sections were incubated in darkness for 10 min. with 3-3′ dianobenzidine tetrahy-
drochloride (DAB) (Sigma Chemical Co., St. Louis, MO, USA), in the amount of 100 mg in 200 ml 0.03% hydrogen peroxide in PBS. The nuclear counterstaining was performed by Mayer’s haemalum for 5 min at room temperature; then slides were dehydrated in ascending ethanol, clarified in xylene and mounted with a synthetic medium (Permount). Negative controls included omission of the primary antiserum or replacement of the primary antiserum with normal horse/goat serum. In each of these conditions, no staining was evident. Placental fragments at the third trimester of pregnancy were utilized as positive control tissue for CRH and POMC antisera. An evident reactivity for POMC and CRF was encountered in syncyto-cytotrophoblast cellular layers of placental tissue.

Immunohistochemical localization of all somatostatin receptors (SSTRs) subtypes was performed on 5 µm paraffin-embedded parallel sections with the polyclonal antibodies against human SSTR₁, SSTR₂α, SSTR₂β, SSTR₃, SSTR₄, SSTR₅ (all from Biotrend, Cologne, Germany; wd: 1: 3500). In brief, the sections were pre-treated in microwave oven in 10 mM citric acid, pH 6.0 (3 cycles for 15 min at 100°C), and incubated over night (o.n.) at 4°C with each antibody. A standard streptavidin-biotin-peroxidase (LSAB² System-HRP multilink universal kit from DAKO) was used according to the manufacturer’s instructions to visualize the bound antibodies, and the reaction was developed in DAB. Negative controls for immunochemistry included: 1) omission of the primary antibody and 2) pre-absorption of the antibodies with the respective immunizing receptor peptide. Peptide fragments of human SSTR₅ subtypes, SSTR₁, SSTR₂α, SSTR₂β, SSTR₃, SSTR₄, SSTR₅ C-term (all from Biotrend, Cologne, Germany), were used at a concentration of 100 nM, and incubated o.n. at 4°C. A tissue was considered positive when the immunostaining was abolished by pre-absorption of the antibody with the respective peptide antigen. Pancreatic tissue sections were utilized as positive controls for SSTR₅ subtypes antisera.

Results

Histological examination

A 7.8x4.8x5.0 cm adrenal mass with a smooth grey-yellowish appearance was excised. The cut surface showed foci of necrosis and haemorrhage as well as areas of cystic degeneration, with a not uniform consistency. The tumour tissue was arranged in sheets, cords or alveolar structures constituted by neoplastic elements showing round vesicular nuclei, occasionally pleomorphic, and a granular, pale eosinophilic, cytoplasm (Figure 2 A), in accordance with the diagnosis of pheochro-
mocytoma. Mitoses were rare and the MIB-1 immunostaining was evident in less than 5% of neoplastic cells. There was no diffuse growth pattern, cellular atypical mitotic figures, or other signs of malignancy.

**Immunohistochemical characterization**

Heterogeneous intensity of positive staining was appreciable with anti-chromogranin A (Figure 2 B) and anti-neurofilaments, neuroendocrine tumour markers that are typically expressed by PHEO. A weak, occasional cytoplasmic immunopositivity was observed also with S-100 antiserum. An intense, mainly diffuse and homogeneous, cytoplasmic immunoreactivity for CRH was found in the tumour, with a high percentage of stained cells (>70%)(Figure 2 C), thus indicating unequivocally that adrenal hyperactivity was CRH dependent, provided that, in contrast with increased serum ACTH levels, no immunostaining for this hormone was apparent (Figure 2 D). Only a weak immunoreactivity for the ACTH precursor POMC was found but concerned a low percentage of cells (<30%) and was not uniform, positive cells being in contact with less reactive or negative ones. The immunohistochemical evaluation of SSTRs expression revealed a positive reaction for SSTR$_1$, SSTR$_{2A}$, SSTR$_{2B}$, sst$_1$, sst$_{2A}$, sst$_{2B}$ on tumour cells (Figure 3). The immunostaining was granular or diffuse, located in the cytoplasm of the neoplastic elements, and the intensity of the reaction was moderate to strong. A membranous staining was also present for the SSTR$_{2A}$ subtype (Figure 3 B). In all cases, immunostaining could be completely abolished by pre-absorption with 100 nM of the respective peptide antigens. The other SSTR subtypes were unreactive.

**Discussion**

The immunohistochemical expression of SSTR$_1$ and SSTR$_{2A}$ and SSTR$_{2B}$ subtypes in the tumour cells demonstrated in the tumour of the studied patient, is correlated with clinical and functional imaging data ($[^{111}]$In-DTPA$_5$ octreotide scintigraphy) along with the effect of somatostatin analogue treatment and the outcome of the disease.

SSTRs is a known characteristic of phaeochromocytomas (De Herder and Hofland, 2004), but information on the specific SSTR$_1$ subtypes determination is lacking. Up to date, few data on the expression of SSTR$_1$ subtypes are available in the literature (Reubi et al., 1992; Kubota et al.,...
1994; Epelbaum et al., 1995; Hofland et al., 1999; Mundschenk et al., 2003; Unger et al., 2004; Ueberberg et al., 2005; Unger et al., 2008).

Reubi et al (1992) for the first time demonstrated the presence of specific somatostatin receptors in phaeochromocytomas. They examined the tumour tissue with regard to the distribution of SSTR2a receptors, and found that 16 of the 18 paraffin-embedded phaeochromocytomas were stained positive for this SSTR subtype (Reubi et al., 1992). Kubota and co-workers (1994), detected SSTR1 and SSTR5 mRNAs in three cases of phaeochromocytoma, showing an expression pattern identical to that of normal adrenal gland. Similar results were obtained by Hofland et al. (1999), who found four of five phaeochromocytomas positive for sst1 and three of five positive for SSTR2a.

Although the mentioned studies demonstrated only the expression of SSTR1 and SSTR5, other Authors investigated the expression pattern of all five SSTR subtypes in benign and malignant phaeochromocytomas. The over-expression of more than one SSTR subtype (other than the sst1), such as SSTR1, SSTR3, SSTR4, and/or sst5 receptor subtypes was identified with a prevalence higher than that of SSTR which were found in less than 50% of these tumours (Epelbaum et al., 1995; Mundschenk et al., 2003; Unger et al., 2004; Unger et al., 2008). Epelbaum and co-workers (Epelbaum et al., 1995) demonstrated in 34 both benign and malignant phaeochromocytomas that mRNAs of all five SSTR subtypes were measurable in the tumour cells as well as in normal adrenal tissue, the SSTR1 and SSTR5 being the most expressed. Indeed, the vast majority (up to 90%) of the phaeochromocytomas examined by Mundschenk and Unger showed positive immunohistochemical staining for SSTR5 in a high percent (>60%) of tumour cells. Immunoreaction for SSTR1, SSTR2a, and SSTR5 receptors was found in only few tumours and with a lower expression within each tumour. Particularly, SSTR2a expression was seen in less than 30% of phaeochromocytomas (Mundschenk et al., 2003; Unger et al., 2004; Unger et al., 2007). These observations were partially confirmed by Ueberberg et al. (2005), who investigated the expression of the five SSTRs in various adrenal tumours and in normal adrenal gland by RT-PCR. They found that each receptor subtype was expressed in normal adrenal gland and in more than 50% of all phaeochromocytomas analyzed, with the SSTR1 being present in nearly all them.

Taken together, these data demonstrate that SSTR subtypes are expressed in phaeochromocytomas with tumor-specific distribution patterns, and the functional responses to somatostatin analogues depend on a mixed population of receptor. Unlike classic neuroendocrine tumors, particularly gastroenteropancreatic tumors, characterized by SSTR2 over-expression, phaeochromocytoma may over-express other receptor subtypes, such as SSTR1 or SSTR5 or SSTR6, for which conventional somatostatin analogs have lower affinity. For this reason, octreotide treatment induced symptomatic or hormonal improvements only in few cases (Invitti et al., 1993; Plouin et al., 1995; Kopf et al., 1997; Lamarre-Cliche et al. 2000), suggesting that octreotide is of limited value for the treatment of this defined group of patients. It may be speculated, that other somatostatin analogues – such as pasireotide/SOM230, which has a broader spectrum than octreotide - may appear to be more efficient. Indeed, in vitro studies have shown inhibition of cell proliferation, induction of apoptosis and reduction of catecholamine secretion (Pasquali et al., 2008), but clinical studies are necessary to confirm these effects.

Nevertheless, a number of pheochromocytomas express the SSTR1 receptor and are able to uptake the radiotracer during octreotide scintigraphy, representing potential target of treatment with somatostatin analogs. In our patient, the SST analog octreotide was able to control hormone hyper secretion and to improve the patient’s general condition. The effectiveness of octreotide treatment was related to the expression of SSTR receptors (mainly the SSTR2a subtype), as preoperatively suggested by an octreoscan performed before starting therapy and demonstrated postsurgically by immunohistochemistry.

Our report confirms the heterogeneity of the pattern of SSTR expression in phaeochromocytomas, and provide further evidence for expression of functional SSTR subtype SSTR2a in a subgroup of phaeochromocytomas. The SST analog octreotide can effectively control symptoms resulting from excessive hormone release and may be performed to achieve a rapid control of the dis-
ease before surgery in such patients. Diagnostic imaging with the $[^{111}\text{In-DTPA}]$ octreotide scan can demonstrate the presence of this specific receptor subtype on these tumours and may help to identify those patients who will have a response to octreotide therapy.

Finally, in our patient, who represent an unprecedented clinical case, the CRH production by the phaeochromocytoma was revealed by the immunohistochemical analysis, that demonstrated a very strong staining for CRH in the tumour cells, in the complete absence of ACTH staining. The CRH secretion by the tumour induced pituitary hyperfunction, as demonstrated by the promptly post-surgical recovery of hypothalamic-pituitary-adrenal axis activity, which made unnecessary steroid replacement after adrenalectomy.

**References**


