Expression of somatostatin receptors, dopamine D₂ receptors, noradrenaline transporters, and vesicular monoamine transporters in 52 pheochromocytomas and paragangliomas

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Abstract

While somatostatin receptors (sst), through somatostatin-radiolabeled analogs, are used, mainly in second line, in the diagnosis and treatment of pheochromocytomas (PCC) and paragangliomas (PGL), the clinical significance of dopamine receptor subtype 2 (D₂) in PCC/PGL is unknown. Indeed, radiolabeled dopamine (DA) analogs such as fluorine 18 (¹⁸F)-DA, used for positron emission tomography in PCC localization, are mainly correlated to the presence of noradrenaline transporter (NAT) and vesicular monoamine transporters (VMAT) but not to D₂. The aim of this study was to quantitate D₂ and sst expression in 52 PCC/PGL and to compare it with that of 35 gastroentero-pancreatic neuroendocrine tumors (GEP-NETs). Quantitative RT-PCR of sst₁–₃ and D₂, NAT, VMAT1/2 was performed in all tumors, while immunohistochemistry analysis of sst₂ and D₂ was performed in seven tumors. D₂ mRNA was expressed in all PCC/PGL. Mean expression was significantly higher in PCC/PGL than in GEP-NETs (4.8 vs 0.5 copy/copy β-glucuronidase (Gus)). sst₂ and sst₁ were expressed in most PCC/PGL, with sst₂-dominant expression (mean mRNA: 1.6 vs 0.4 copy/copy β-Gus). sst₂ expression level was similar to that of GEP-NETs, whereas sst₁ expression level was significantly lower (0.12 vs 0.78 copy/copy β-Gus). Our study evidenced strong D₂ mRNA expression in PCC and for the first time in PGL. PCC/PGL express sst₂ mRNA at levels similar to those of GEP-NETs. New drugs can target ssts and D₂ more efficiently than current somatostatin analogs. Moreover, transporters like NAT and VMAT1/2, could be co-targeted with sst, as a basis of new radionuclide compounds in the imaging and treatment of these tumors.

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Introduction

Pheochromocytomas (PCC) and paragangliomas (PGL) are neuroendocrine tumors derived from adrenal chromaffin cells and extra-adrenal paraganglia respectively (Eisenhofer et al. 2008). These tumors cause variable secondary hypertension, often difficult
to control, with a lethal potential. Up to 26% of PCC/PGL are malignant (Ilias & Pacak 2008). Two specific radio nuclide-based approaches in diagnosis and treatment have been developed. One is based on meta-iodobenzylguanidine (MIBG), a biogenic amine analog taken up by membrane noradrenaline transporter (NAT) and concentrated in tumors via vesicular monoamine transporters (VMAT). This molecule is labeled by $^{123}$I for diagnosis and $^{131}$I for diagnosis and treatment (Kolby et al. 2003, Cleary & Phillips 2006, Martiniova et al. 2006). The second approach is based on somatostatin (SRIF) receptor radioligands (Reubi et al. 2000a).

SRIF exerts inhibitory effects on endocrine secretion and proliferation of many endocrine tumors (Reubi & Laissue 1995). SRIF analogs (octreotide and lanreotide) are widely used in the treatment of pituitary GH tumors and gastroenteropancreatic neuroendocrine (GEP-NETs) (Invitti et al. 1993, Kopf et al. 1997, Feelders et al. 2009, Oberg et al. 2009). The various effects of somatostatin and its analogs are mediated through five-membrane G protein-coupled receptors: somatostatin receptor subtypes 1–5 (sst$_{1–5}$). The presence of sst in PCC/PGL has been demonstrated by ligand binding studies (Epelbaum et al. 1995, Reubi & Laissue 1995, Reubi et al. 2001), by immunohistochemistry (Mundschenk et al. 2003, de Herder & Hofland 2004, Unger et al. 2004), and more recently by reverse transcriptase (RT)-PCR (Ueberberg et al. 2005, Kolby et al. 2006, Binderup et al. 2008). However, the current SRIF analogs, directed mainly to sst$_3$, have little if any efficacy on catecholamine secretion of PCC (Invitti et al. 1993, Plouin et al. 1995, Kopf et al. 1997, Lamarre-Cliche et al. 2002), or on tumoral growth of PGL (Tonyukuk et al. 2003). Two new somatostatin analogs with different binding profiles are in advanced clinical trials for various endocrine tumors: pasireotide, an sst ‘panagonist’ and BIM-23A760 dopastatin, with strong affinity for sst$_2$ and dopamine receptor subtype 2 (D$_2$). The D$_2$ affinity of dopastatin contributes strongly to the effect of this molecule in pituitary tumors (Pupilli et al. 1994, Saveanu et al. 2002, Jaquet et al. 2005). DR and in particular D$_2$ have been identified in PCC at mRNA and protein level (Pupilli et al. 1994, Wu et al. 2001, Pivonello et al. 2004). D$_2$ antagonists modify catecholamine secretion (Mannelli et al. 1988), whereas the D$_2$ agonists were not yet directly tested on PCC secretion to our knowledge.

The aim of this study was to quantitate D$_2$ and sst mRNA expression in a large group of PCC/PGL in comparison with GEP-NETs. GEP-NETs were chosen as they share with PCC/PGL neural crest origin and some radiolabeled diagnosis methods and treatment. Moreover, we looked for correlations between D$_2$/sst expression and transporter mRNA (NAT/VMAT) expression in order to propose new diagnostic and therapeutic approaches using multiple peptide targeting.

Materials and methods

PCC and PGL tumors

The study was carried out on 43 patients (20 men and 23 women), aged 46 ± 19 year bearing at least one PCC or PGL tumor. This study was approved by the ethics committee of the University and undertaken after informed consent was obtained from each patient. All tumors had been surgically removed and pathological characteristics were available. Of the 52 tumors included, 39 were PCC (one malignant, PCC31) and 13 PGL (Supplementary Table 1, see section on supplementary data given at the end of this article). After surgery, a portion of each tumor tissue was frozen for further analysis.

In five cases, the PCC appeared in a multiple endocrine neoplasia type 2 (MEN2) context with a RET mutation. Two patients had a neurofibromatosis with NFI mutation and one of them presented a bilateral PCC (PCC32 and PCC33). For the 36 remaining patients, SDHB, SDHD, and VHL analysis was performed by direct sequence analysis from whole blood leucocytes as described previously (Taieb et al. 2009). Among these 36 patients, four had a mutation on SDHD, two on SDHB, and two on VHL. In these patients, multiple tumors were frequent: one SDHD patient with two PCC (PCC7 and PCC8) and one VHL patient with PCC (PCC28) and PGL (PGL3) (Supplementary Table 1, see section on supplementary data given at the end of this article). In total, ten patients (16 tumors) were screened before surgery by $^{111}$In-pentetreotide scintigraphy, whereas 35 patients (46 tumors) were screened by $^{131}$I-MIBG scintigraphy (Table 1). In particular, one SDHD patient presented with seven tumoral localizations (Table 2) and was screened before surgery by $^{18}$F-labeled deoxyglucose ($^{18}$F-FDG) positron emission tomography (PET), $[^{18}$F] dihydroxyphenylalanine ($^{18}$F-DOPA) PET, $^{111}$In-pentetreotide scintigraphy, and $^{131}$I-MIBG scintigraphy (Taieb et al. 2008). Quantitative expressions of mRNA for receptors targeted by somatostatin and dopamine agonists (sst$_{1–3}$, sst$_5$ and D$_2$) were compared with those obtained in a group of 35 GEP-NETs previously characterized.
We used the 5′-exonuclease (TaqMan) assay, which produces a direct proportional readout for the progression of PCR. Amplification of cDNA derived from 50 ng total RNA was performed in a 25 μl reaction volume with 300 nM of each primer, 200 nM of the probe, and 12.5 μl MasterMix (PE Applied Biosystems, Paris, France). The synthetic sst₁, sst₂, sst₃, sst₅, D₂ primers, and TaqMan probes used in the PCR were described previously (O'Toole et al. 2006). The primers and probes for VMAT₁, VMAT₂, and NAT were purchased from Applied Biosystems as TaqMan gene expression assays ID: Hs00161839, Hs00161858, and Hs00426573, respectively (Applied Biosystems). sst₄ was not assessed, although infrequent in these tumor types and not targeted by current somatostatin analogs.

We performed 40 cycles of two-step PCR annealing-extension on an ABI Prism 7700 sequence detection apparatus (PE Applied Biosystems). The mRNA levels were normalized to the β-glucuronidase (β-Gus) mRNA levels obtained in the same reaction (O'Toole et al. 2006). The β-Gus primers and probe were purchased from PE Applied Biosystems. Two other reference genes were assessed: β₂-microglobulin and β-actin (primers and probes purchased from Applied Biosystems, references at request). For each measurement, three independent PCR analyses were performed. To produce standard curves for sst, D₂, VMAT₁, VMAT₂, NAT, and β-Gus mRNA, cDNA plasmid constructs were produced for each parameter and verified by sequencing. The results were expressed as copy of mRNA of gene of interest/copy of mRNA of β-Gus, in order to allow comparison with previous published data on pituitary and GEP-NETs. β-Gus levels in PCC/PGL were stable. The normalization with the three reference genes does not change the overall results (data not shown).

### Table 1 Patients and tumors characteristics

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>n=43</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender male/female</td>
<td>20/23</td>
</tr>
<tr>
<td>Age, mean (range) years</td>
<td>46 (14–74)</td>
</tr>
<tr>
<td>Number of tumors</td>
<td>39 13</td>
</tr>
<tr>
<td>Tumour size (mean ± s.e.m.)</td>
<td>47 ± 29 30 ± 25</td>
</tr>
<tr>
<td>Hereditary/sporadic</td>
<td>13/26</td>
</tr>
</tbody>
</table>

### Table 2 Comparison between mRNA expression levels of sst₂, D₂, VMAT₁, VMAT₂, and NAT and in vivo functional imagery of 2 PCC and 5 abdominal PGL from 1 patient bearing a SDHD mutation. mRNA receptor results are expressed in copy/copy β-Gus

<table>
<thead>
<tr>
<th>PGL/PCC</th>
<th>Tumor diameter (mm)</th>
<th>sst₂</th>
<th>D₂</th>
<th>VMAT₁</th>
<th>VMAT₂</th>
<th>NAT</th>
<th>SRS</th>
<th>¹³¹I-MIBG</th>
<th>¹⁸F-DOPA</th>
<th>¹⁸F-FDG</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGL 10</td>
<td>12</td>
<td>0.51</td>
<td>1.17</td>
<td>20</td>
<td>15</td>
<td>3.29</td>
<td></td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PGL 11</td>
<td>17</td>
<td>0.12</td>
<td>2.36</td>
<td>85</td>
<td>41</td>
<td>2.67</td>
<td></td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>PGL 12</td>
<td>6</td>
<td>0.34</td>
<td>2.99</td>
<td>67</td>
<td>54</td>
<td>3.84</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PGL 4</td>
<td>9</td>
<td>0.62</td>
<td>1.73</td>
<td>23</td>
<td>16</td>
<td>1.40</td>
<td></td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>PGL 5</td>
<td>25</td>
<td>1.14</td>
<td>3.72</td>
<td>144</td>
<td>109</td>
<td>5.27</td>
<td></td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PCC 29</td>
<td>9</td>
<td>0.61</td>
<td>2.52</td>
<td>37</td>
<td>59</td>
<td>1.74</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PCC 30</td>
<td>48</td>
<td>1.17</td>
<td>7.01</td>
<td>74</td>
<td>62</td>
<td>14.75</td>
<td></td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

SRS, somatostatin receptor scintigraphy (¹¹¹In-pentetreotide).

(ÓToole et al. 2006). GEP-NETs mRNA was analyzed again, in the same series with PCC, in order to avoid inter-experiments variation.

Detection of sst₁, sst₂, sst₃, sst₅, D₂ receptors, and monoamine transporters (VMAT₁, VMAT₂, and NAT) mRNAs

Total RNA was extracted from 30 to 60 mg of tissue from each tumor by the RNA easy isolation system (Qiagen). Tissue samples were carefully evaluated at microscopy to ensure that sampling was from tumoral tissue and not from adjacent tissues. In four cases, normal peritumoral adrenal medulla tissue was also used for mRNA extraction (PCC9, PCC23, PCC28, and PCC29). Total RNA (1 μg) prepared from tumoral or normal tissues was used for cDNA synthesis with 200 U Superscript II reverse transcriptase (Life Technologies, Inc., Cergy-Pontoise, France) primed with 300 ng random primer (O'Toole et al. 2006).
**sst2 and D2 immunohistochemistry analysis**

To correlate sst2 and D2 mRNA expression with the corresponding receptor protein, an immunohistochemistry analysis was performed on seven available tumors, two PGL and five PCC (Table 3). Tumor specimens were previously fixed in formalin and embedded in paraffin. Normal peritumoral endocrine pancreatic tissue and normal adrenal medulla were used as positive control of sst2 presence. A prolactin secreting pituitary adenoma was used as positive control of D2 presence. Immunostaining for sst2 was performed using polyclonal rabbit antibodies raised against a sequence in the carboxyterminal region of the sst2A receptor protein (SS-800; Gramsch Laboratories, Schwabhausen, Germany) diluted at 1:2000. Immunostaining for D2 was performed using monoclonal mouse antibodies raised against a sequence in the aminoterminal region of the D2 receptor protein (SC-5303; Santa Cruz Biotechnology, Heidelberg, Germany) diluted at 1:100. In both cases, the reaction was performed using an automated immunoperoxidase procedure in the Ventana Benchmark device (Ventana kit, Tucson, AZ, USA).

The intensity of immunostaining was scored with the following semiquantitative method: 0 absent, + weak, ++ moderate, and +++ strong immunostaining. Moreover, cytoplasmic or membrane localization was specified for sst2.

**Statistical analysis**

The results are presented as the mean ± s.e.m. Statistical significance between two unpaired groups was determined by the Mann–Whitney U test. To measure the strength of association between pairs of variables without specifying dependency, the Spearman rank order correlations were run. A P value lower than 0.05 was considered significant for all tests.

**Results**

**sst and D2 mRNA expression in PCC and PGL**

All PCC and PGL expressed D2 mRNA (Table 4 and Fig. 1A). In 72% of tumors, the level of D2 was higher than 2 copy/copy β-Gus. The D2 mean mRNA levels were significantly higher in PCC than in PGL (P < 0.002, Table 4). In hereditary tumors, VHL tumors presented the lowest D2 expression (1.9 ± 1 copy/copy β-Gus), followed by SDH tumors (2.3 ± 1 copy/copy β-Gus), whereas NEM2 tumors and sporadic tumors presented a similar higher mean D2 mRNA (6.0 ± 1 and 5.9 ± 0.8 copy/copy β-Gus, respectively). However, differences did not achieve statistical significance, due to the low number of cases in each group of hereditary tumors.

Among ssts, sst2, and sst1 were the main subtypes, widely expressed in 100% and 94% of tumors, respectively. Mean sst2 mRNA level was higher than that of sst1 mRNA (1.6 ± 1.5 vs 0.6 ± 0.9 copy/copy β-Gus). sst2 mRNA level was higher than sst1 mRNA level in 90% of analyzed tumors. There was no statistically significant difference between PCC and PGL concerning the expression of these two receptors (see Table 4).

sst3 was expressed in 53% of PCC/PGL, and sst5 was expressed in only 47% of PCC/PGL; mean mRNA level was low in both cases (see Table 4). However, the two receptors were more significantly expressed in PCC than in PGL tumors (Table 4). There were

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**Table 3** Comparison of immunohistochemistry and mRNA expression levels for sst2 and D2 in seven PCC/PGL tumors

<table>
<thead>
<tr>
<th>Tumors</th>
<th>sst2 mRNA</th>
<th>Whole</th>
<th>Membrane</th>
<th>Cytoplasmic</th>
<th>D2 mRNA</th>
<th>D2 immunohistochemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCC9</td>
<td>3.59</td>
<td>+</td>
<td>++</td>
<td>0</td>
<td>12.20</td>
<td>+/+/+b</td>
</tr>
<tr>
<td>PCC23</td>
<td>2.25</td>
<td>+/+</td>
<td>++</td>
<td>+/+/a</td>
<td>9.02</td>
<td>+/+</td>
</tr>
<tr>
<td>PCC25</td>
<td>0.79</td>
<td>+</td>
<td>+/−/a</td>
<td>+</td>
<td>3.21</td>
<td>+</td>
</tr>
<tr>
<td>PCC28</td>
<td>0.94</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2.05</td>
<td>+/+/a+b</td>
</tr>
<tr>
<td>PCC29</td>
<td>0.61</td>
<td>+</td>
<td>+/−/a</td>
<td>+</td>
<td>2.52</td>
<td>+</td>
</tr>
<tr>
<td>PGL1</td>
<td>0.85</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1.08</td>
<td>0</td>
</tr>
<tr>
<td>PGL5</td>
<td>1.14</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>3.72</td>
<td>+</td>
</tr>
<tr>
<td>Normal adrenal</td>
<td>1.16+/−0.2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>medulla</td>
<td>(n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

mRNA results, quantified by real-time PCR, are expressed as mean ± s.e.m. copy/copy β-Gus. ND, not done.

aFocal localization.

bHeterogenous localization.
no significant differences in sst$_{1-3}$, and sst$_5$ between sporadic and different hereditary tumor types (data not shown). Although there was no correlation between sst$_2$ and $D_2$ mRNA expression, the mRNA levels of $D_2$ mRNA were strongly correlated to those of sst$_1$ in both types of tumors (Fig. 1B, $r=0.46$, $P < 0.0009$).

**Comparison of sst and $D_2$ mRNA levels between PCC/PGL and GEP-NETs**

Somatostatin receptor subtypes sst$_{1-3}$, and sst$_5$ and $D_2$ mRNA levels in PCC and PGL were compared with those in a group of 35 GEP-NETs (Fig. 2 and Table 4). Mean levels of $D_2$ mRNA were significantly higher in PCC and PGL than in GEP-NETs ($P < 0.0001$). Surprisingly, sst$_2$ mRNA levels were similar in both tumor groups (Table 4 and Fig. 2). sst$_5$ mRNA mean levels were more than five times lower in sst$_5$ expressing PCC and PGL than in sst$_5$ expressing GEP-NETs (0.14 vs 0.08 vs 0.78 copy/copy β-Gus, $P < 0.0001$ and $P < 0.04$, respectively Table 4 and Fig. 2). Finally, sst$_1$ and sst$_3$ mRNA mean expression was low and similar in PGL and GEP-NETs, whereas slightly but significantly higher for sst$_1$ in PCC (Table 4). There was no significant difference in sst$_2$ or sst$_5$ mRNA between $^{111}$In-pentreotide-positive ($n=4$) and -negative tumors ($n=12$; data not shown). In control normal endocrine cells of pancreas, a diffuse positive reaction was found in cytoplasmics, whereas membrane staining, present in occasional cells, was less conspicuous (Fig. 3A). On the contrary, staining in normal adrenal medulla cells was more contrasted but almost exclusively concentrated along cytoplasmic membranes (Fig. 3B).

**sst$_2$ immunohistochemistry analysis**

Considering the strong unexpected sst$_2$ mRNA expression in PCC/PGL, the presence of sst$_2$ protein was also analyzed also by immunocytochemistry in seven PCC/PGL.

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**Table 4** Expression mRNA levels of sst$_1$, sst$_2$, sst$_3$, sst$_5$, $D_2$, VMAT1, VMAT2, and NAT in PCC, PGL and GEP

<table>
<thead>
<tr>
<th></th>
<th>PCC (n=39)</th>
<th>PGL (n=13)</th>
<th>PCC/PGL</th>
<th>GEP-NETs (n=35)</th>
<th>PCC/ GEP-NETs</th>
<th>PGL/ GEP-NETs</th>
</tr>
</thead>
<tbody>
<tr>
<td>sst$_1$</td>
<td>0.53 ± 0.13 (95%)</td>
<td>0.16 ± 0.05 (92%)</td>
<td>NS</td>
<td>0.07 ± 0.01 (100%)</td>
<td>$P &lt; 0.0001$</td>
<td>NS</td>
</tr>
<tr>
<td>sst$_2$</td>
<td>1.68 ± 0.23 (100%)</td>
<td>1.37 ± 0.32 (100%)</td>
<td>NS</td>
<td>1.53 ± 0.2 (100%)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>sst$_3$</td>
<td>0.30 ± 0.01 (56%)</td>
<td>0.08 ± 0.04 (31%)</td>
<td>$P &lt; 0.02$</td>
<td>0.09 ± 0.03 (63%)</td>
<td>$P &lt; 0.0006$</td>
<td>NS</td>
</tr>
<tr>
<td>sst$_5$</td>
<td>0.14 ± 0.09 (54%)</td>
<td>0.08 ± 0.05 (31%)</td>
<td>$P &lt; 0.03$</td>
<td>0.78 ± 0.21 (89%)</td>
<td>$P &lt; 0.0001$</td>
<td>$P &lt; 0.04$</td>
</tr>
<tr>
<td>$D_2$</td>
<td>5.72 ± 0.6 (100%)</td>
<td>1.92 ± 0.38 (100%)</td>
<td>$P &lt; 0.0002$</td>
<td>0.50 ± 0.21 (100%)</td>
<td>$P &lt; 0.0001$</td>
<td>$P &lt; 0.0001$</td>
</tr>
<tr>
<td>VMAT1</td>
<td>28.7 ± 7 (100%)</td>
<td>42 ± 11 (100%)</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VMAT2</td>
<td>35.4 ± 7 (100%)</td>
<td>41.5 ± 7 (100%)</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAT</td>
<td>5.9 ± 0.7 (95%)</td>
<td>2.3 ± 0.4 (77%)</td>
<td>$P &lt; 0.004$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NS = not significant. The percentage of tumors expressing a receptor subtype are indicated in parentheses. Results are expressed as mean ± s.e.m. copy/copy β-Gus

*Comparison between PCC and PGL using Mann–Whitney U test.*

*Comparison between PCC and GEP-NETs using Mann–Whitney U test.*

*Comparison between PGL and GEP-NETs using Mann–Whitney U test.*
In the seven analyzed PCC/PGL tumors (Table 3), the expression of sst2 protein was highly variable, from a lack of immunostaining (Fig. 3C) to a strong reaction (Fig. 3D). The sst2 immunoreactivity was principally located on the cytoplasmic membranes (Fig. 3D) and to a lesser extent in cytoplasms (Fig. 3E). In most cases it was homogeneous, but sometimes heterogeneous (Fig. 3F). Contrasted and diffuse membrane staining was found in two tumors containing the largest amount of sst2 mRNA receptor according to Q-PCR analysis.

**Figure 2** Comparison of mRNA expression levels of sst2 (left panel), sst5 (middle panel), and D2 (right panel) between 39 PCC (filled circles), 13 PGL (filled triangles) and 35 GEP-NETs (open circles). The quantification was performed by real-time PCR. Measurements were normalized to the level of β-Gus mRNA (copy/copy β-Gus). The horizontal bar represents the mean. *P<0.05, **P<0.01, ***P<0.0001, NS, not significant.

**Figure 3** Immunohistochemical analysis of sst2 receptor expression. (A) Diffuse cytoplasmic and membrane staining in normal pancreatic endocrine islet (400×). (B) Sharp membrane staining in normal adrenal medullary cells (200×). (C) Negative reaction in a case of PCC (PCC28) (200×). (D) Homogeneous membrane expression of sst2 in a case of PCC (PCC9) (400×). (E) Mostly granular cytoplasmic expression of sst2 in a case of PGL (PGL1) (400×). (F) Heterogeneous reaction associating areas of membrane and cytoplasmic reaction in a case of PCC (PCC29) (400×). Scale bars: 50 μm (A, D, E, and F); 100 μm (B and C).
(PCC23 and PCC9). Overall, the expression of sst\textsubscript{2} receptor protein varied in intensity: lack of immunostaining (\(n=1\); PCC28), weak immunostaining (\(n=3\); PGL1, PCC25, and PCC29), moderate immunostaining (\(n=2\); PCC9 and PGL5), and strong immunostaining (\(n=1\), PCC23; Table 3 and Fig. 3). The only semiquantitative IHC analysis on one side, and the weak number of cases on the other side, could explain that no statistically significant correlation was found. However, in 6/7 cases, sst\textsubscript{2} mRNA was associated with protein expression.

**D\textsubscript{2} immunohistochemistry analysis**

In control pituitary prolactinoma cells, a diffuse strong granular reaction was found in cytoplasms and along cell membranes (Fig. 4A).

In the seven analyzed PCC/PGL tumors (Table 3), the expression of D\textsubscript{2} protein was highly variable, from a lack of immunostaining (Fig. 4B) to a strong reaction (Fig. 4C). In most cases the staining was homogeneous, but sometimes heterogeneous (PCC9, PCC28, not shown). The expression of D\textsubscript{2} protein varied in intensity: lack of immunostaining (\(n=1\); PGL1), weak/moderate immunostaining (\(n=2\); PCC9 and PCC28), moderate immunostaining (\(n=3\); PGL5, PCC25, and PCC29), strong immunostaining (\(n=1\), PCC23) (Table 3 and Fig. 4). D\textsubscript{2} immunostaining was significantly correlated to the D\textsubscript{2} mRNA expression levels (\(P<0.02\)). However, in PCC9, which presented high D\textsubscript{2} mRNA level, only a weak/moderate D\textsubscript{2} immunostaining was found. This may be partially explained by heterogenous immunostaining in this tumor (data not shown).

**VMAT1, VMAT2, and NAT mRNA expression**

All PCC and PGL expressed at variable VMAT1 and VMAT2 mRNA levels (Table 4 and Fig. 5A). VMAT1 and VMAT2 mRNA levels were strongly correlated in both tumor types (\(P<0.0001\); \(r=0.57\), global, Fig. 5B; \(P<0.0003\) for PCC and \(P<0.02\) for PGL, respectively). Mean transporter mRNA expression was higher in PGL than in PCC for both VMAT1 and VMAT2, but without achieving statistical significance (Table 4). NAT mRNAs were found in 95\% of PCC and 77\% of PGL tumors. NAT mRNA expression was much higher in PCC than in PGL (\(P<0.004\), Table 4). It was correlated to D\textsubscript{2} mRNA expression in both tumor types (\(P<0.0001\), \(r=0.56\), global, Fig. 5C; \(P<0.02\) for PCC and \(P<0.02\) for PGL, respectively). Analysis of hereditary PCC/PGL showed an absent or low expression of NAT mRNA in VHL tumors (PCC27, PCC28, and PGL3, \(P<0.01\) versus sporadic tumors), whereas NAT expression was in the higher range in 4/5 MEN2 tumors, (5.7–15.2 copy/copy β-Gus, Supplementary Table 1, see section on supplementary data given at the end of this article), even if this trend was not validated by a statistically significant difference (versus sporadic tumors). There was no significant difference of VMAT1/2 or NAT mRNA levels between \(^{131}\text{I}-\text{MIBG} \) scintigraphy-positive (\(n=41\)) and -negative (\(n=5\)) tumors (data not shown).
We had the opportunity to compare the sst2, D2, VMAT1, and VMAT2 mRNA levels of seven tumors, five PGL and two PCC, from the same patient bearing a SDHD mutation. The expression of each transcript was highly variable between tumors, in a range of one decimal logarithm (log) for sst2, 0.5 log for D2, 0.7 log for VMAT1 and VMAT2, and 1 log for NAT mRNA (Table 2). In this patient, somatostatin receptor scintigraphy (SRS; 111In pentreotide) was negative. 131I-MIBG scintigraphy identified 4/7 tumors, whereas 18F-DOPA PET imaging identified only three out of seven tumors. 18F-FDG PET imaging was the only imaging technique localizing all 7 tumors (Table 2). The diameter of 131I-MIBG scintigraphy-negative tumors was lower than that of positive tumors, without achieving statistical significance (8 ± 1 vs 25 ± 6 mm, P < 0.06).

Discussion

Our study provides the first quantitative analysis of D2 mRNA expression in a large series of PCC/PGL, evidencing a high expression of this receptor in most tumors (>2 copy/copy β-Gus). The mean D2 mRNA level was similar to that found in pituitary GH and gonadotroph tumors (Saveanu et al. 2006, Florio et al. 2008) but was clearly higher than that of the GEP-NETs. Previous studies have shown the presence of D2 in normal adrenal medulla and PCC by northern blot (Pupilli et al. 1994, Wu et al. 2001), by RT-PCR (Wu et al. 2001), or by RT-PCR, immunohistochemistry, and ligand binding studies (Pivonello et al. 2004). However, these studies concerned a limited number of nine PCC and no PGL. Our data show for the first time that D2 is also expressed in PGL at high levels, although lower than the levels encountered in PCC. The relevance of D2 expression in PCC/PGL is to be further investigated, but it is interesting to note that it correlates to NAT mRNA expression. At membrane level, NAT and D2 may compete for dopamine and D2 agonists. Previous studies have shown that most PCC express NAT (Cleary et al. 2005, Huynh et al. 2005). NAT belongs to the SLC6 family of plasma membrane transporters and acts by rapidly sequestering released biogenic amines (Amara et al. 1998). In neuroendocrine tumors, NAT allows the entry of radiolabeled elements such as 131I-MIBG (Glowniak et al. 1993) and 18F-dopamine (18F-DA) (Martiniova et al. 2006). MIBG accumulation is well correlated to NAT expression in neuroblastomas (Mairs et al. 1994), but data are lacking for PCC. Our quantitative analysis of NAT mRNA showed a high variability of expression, in a range of three decimal logarithms (from 0.01 to 15 copy/copy β-Gus), with a significantly higher expression in PCC than in PGL. In hereditary tumors, note that NAT mRNA expression was particularly low in the three VHL PCC/PGL and higher than mean in 4/5 MEN2 PCC, as described previously (Huynh et al. 2005). The low NAT expression in VHL tumors could explain the lower efficacy of MIBG imaging in detecting these tumors, whether the failure of MIBG
imaging in VHL tumors is contested by others (Srirangalingam et al. 2009).

By transferring dopamine into intracellular storage vesicles, the contribution of VMAT1 and VMAT2 in MIBG, \(^{18}\)\(^{3}\)F-DOPA, and \(^{18}\)\(^{3}\)F-DA imaging is crucial (Eisenhofer et al. 2001, Kolby et al. 2003, Havekes et al. 2009). Indeed, in PCC, MIBG accumulation is well correlated to VMAT expression (Kolby et al. 2003). We found a significant positive relationship of mRNA levels between VMAT1 and VMAT2 in our series of sporadic and hereditary PCC/PGL, in agreement with the data of Huynh et al. 2005 but concerning exclusively hereditary PCC. D\(_2\) and VMAT1/2 coexpression in PCC/PGL also raises the question of a possible interaction between D\(_2\) and VMAT1/2. A recent study in PC12 PCC cell line showed that a D\(_2\) agonist (bromocriptine) displays an inhibitory effect on the \(^{3}\)H dopamine uptake through VMAT1/2 in these cells (Izumi et al. 2008). However, for the rat striatum, other authors (Brown et al. 2001, Truong et al. 2004) showed that another D\(_2\) agonist (quinpirole) stimulates the uptake of \(^{3}\)H dopamine. VMAT1/2 expression is not the only determinant for a positive MIBG/F-DOPA imaging. Indeed in the patient with seven tumor localizations (two PCC and five PGL), tumor diameter was an important factor for positive MIBG imaging, as suggested by others in PCC (Bhatia et al. 2008).

SRS is a second option for detecting PCC and PGL. There is a clear difference between PCC, where SRS sensitivity/specificity is lower than that of MIBG scintigraphy (Tenenbaum et al. 1995, van der Harst et al. 2001, Kaltsas et al. 2001) and head and neck PGL, where SRS sensitivity/specificity is clearly better than that of MIBG scintigraphy (Koopmans et al. 2008). Somatostatin receptors are expressed in both PCC and PGL. Among the five subtypes of sst, our results by quantitative RT-PCR showed a predominant expression of sst\(_2\) in the 52 PCC/PGL. This is in agreement with the data obtained in most immunohistochemistry, ligand binding, or RT-PCR studies (Epelbaum et al. 1995, Hofland et al. 1999, Kimura et al. 1999, Reubi et al. 2000b, Kolby et al. 2006, Binderup et al. 2008). Moreover, we showed that mean levels of sst\(_2\) mRNA are similar to those observed in GEP-NETs, as already suggested by Binderup et al. (2008). However, a lower sst\(_2\) expression was observed in four metastatic PCC/PGL by Kolby et al. (2006). The similar mean sst\(_2\) mRNA level in PCC/PGL and GEP-NETs in our study is intriguing, considering the fact that the current SRIF analogs (octreotide, lanreotide), which are directed mainly to sst\(_2\), have little if any efficacy on catecholamine secretion of PCC (Invitti et al. 1993, Plouin et al. 1995, Kopf et al. 1997, Lamarre-Cliche et al. 2002), or on tumoral growth of PGL (Tonyukuk et al. 2003). sst\(_2\) labeling was clearly observed in 6/7 tumors analyzed by immunohistochemistry, confirming the presence of sst\(_2\) protein in our series. However, the cytoplasmic localization of the sst\(_2\) receptor in some tumors might partially explain the failure of sst\(_2\) agonists in controlling catecholamine secretion and tumor proliferation, or the failure of SRS imaging to detect some PCC/PGL, as previously suggested by others (Reubi et al. 2000b). Further functional studies are needed to assess the failure of sst\(_2\) agonists in controlling PCC/PGL secretion and proliferation. In our study, the main difference in sst expression pattern between PCC/PGL and GEP-NETs concerns sst\(_5\). sst\(_5\) was found weakly expressed in <50% of PCC/PGL, levels clearly lower than in the two types of tumors efficiently targeted by current SRIF agonists, GEP-NETs (O'Toole et al. 2006) and GH pituitary tumors (Saveanu et al. 2001, 2006). The comparison of sst expression profile between endocrine tumors suggest a role for sst\(_5\) subtype in the response to the current SRIF analogs (octreotide and lanreotide), which recognize mostly sst\(_2\), but have a slight affinity for sst\(_5\) too. Indeed, sst\(_2\) and sst\(_5\) were reported to heterodimerize (Grant et al. 2008), sst\(_2\) and sst\(_5\) act additively on GH secretion (Saveanu et al. 2001), and sst\(_5\) presence drastically modifies sst\(_2\) internalization, maybe affecting cellular desensitization to somatostatin (Sharif et al. 2007). Most previous studies have found similar results for sst\(_5\), with low if any expression in more than 100 investigated PCC (Reubi et al. 2001, Mundschken et al. 2003, Unger et al. 2004, Ueberberg et al. 2005, Kolby et al. 2006, Binderup et al. 2008). Only one study reported significant sst\(_5\) expression in all six investigated PCC (Pasquali et al. 2008).

sst\(_1\) is constantly expressed in PCC/PGL, at lower levels than sst\(_2\), as previously reported by other studies on a lower number of cases (Kubota et al. 1994, Kolby et al. 2006, Binderup et al. 2008). Finally, we found sst\(_3\) mRNA expression in only half of PCC/PGL, at low levels, in agreement with previous RT-PCR studies (Ueberberg et al. 2005, Binderup et al. 2008, Pasquali et al. 2008). However, other authors identified sst\(_3\) in most analyzed PCC by immunohistochemistry (Mundschken et al. 2003, Unger et al. 2004).

sst expression in PCC/PGL may be of interest for both imaging and therapeutics. As already mentioned for PGL, SRS with \(^{111}\)In-pentetreotide is a first-line detection tool with high sensitivity/specificity (Schmidt et al. 2002, Duet al. 2003, Koopmans et al. 2008). However, in PCC, its sensitivity is clearly lower than that of \(^{18}\)\(^{3}\)F-DA PET and MIBG scintigraphy.
(van der Harst et al. 2001, Ilias et al. 2008). New $^{68}$Ga-labeled octreotide derivatives used for PET imaging have improved the sensitivity in detection of PCC compared with $^{111}$In-pentetreotide (Gabriel et al. 2007), making somatostatin receptor PET imaging a useful complementary technique in PCC/PGL imagery. Moreover, sst-based radiation therapy by Lutetium ($^{177}$Lu)- or Yttrium ($^{90}$Y)-radionabeled octreotide derivatives, currently used in GEP-NETs (Kwekkeboom et al. 2010), can be applied to metastatic PCC/PGL with good efficacy in selected cases (van Essen et al. 2006, 2007, Forrer et al. 2008).

Pasireotide (SOM-230), by targeting sst$_1$, sst$_2$, sst$_3$, and sst$_5$, showed better efficacy than octreotide in the suppression of ACTH and GH secretion from pituitary tumor cells (van der Hoek et al. 2004, de Bruin et al. 2009, Petersenn et al. 2010). In PCC-cultured cells, pasireotide also showed a greater inhibitory effect than octreotide on catecholamine secretion (Pasquali et al. 2008). This better inhibition by pasireotide in PCC may be explained either by the broader affinity of this molecule for ssts or by its longer halftime (Weckbecker et al. 2002). The constant co-expression of sst$_1$ and sst$_2$ found in our series suggests that pasireotide effect may pass through sst$_1$, rather than sst$_5$ or sst$_3$, in this type of tumor. Furthermore, the coexpression of ssts and of D$_2$ in PCC/PGL suggests that new chimeric somatostatin and dopamine analogs (dopastatins; Jaquet et al. 2005) may also be used as backbone for the construction of radionabeled analogs for PCC/PGL imagery and treatment.

Transporters and receptors studied here may be co-targeted by new innovative drugs. Indeed, the uptake of a $^{131}$I-MIBG-octreotate conjugate drug was shown to be higher than that of an iodine octreotide analog in a human neuroblastoma cell line SK-N-SH transfected with sst$_2$. The authors suggested a complementary role from NAT/VMAT transporters added to a predominant sst$_2$ contribution (Vaidyanathan et al. 2007).

In conclusion, we demonstrated that PCC and for the first time PGL both express high levels of D$_2$ mRNA, clearly higher than GEP-NETs. Moreover, PCC/PGL co-express somatostatin receptors sst$_1$ and sst$_2$ at high levels. Receptor expression is associated with high transporter expression: NAT and VMAT1/2.

Functional studies are needed to better understand the relevance of different sst and D$_2$ expressed in PCC/PGL. However, in a multiple peptidic target approach in cancer (Reubi & Waser 2003, Jaggi et al. 2008), co-targeting receptors expressed in PCC/PGL by multispecific agonists, as well as conjugated somatostatin analogs to MIBG, may open new ways for radionuclide compounds with a wider spectrum in the imaging and treatment of PCC/PGL.

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-10-0175.

**Declaration of interest**

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

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