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 gastroenteropancreatic neuroendocrine tumors (GEP-NETs). Quantitative RT-PCR of sst₁–₃ and sst₅, 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$_2$, have little if any efficacy on catecholamine secretion of PCC (Invitti et al. 1993, Plouin et al. 1995, Kopf et al. 1997, Lamarre-Ciche 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 adrenal 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 NF1 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...
(O’Toole 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).

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, 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>
<tr>
<td>MEN2/RET = 5</td>
<td>SDHB = 1</td>
</tr>
<tr>
<td>SDHB = 1</td>
<td>SDHD = 9</td>
</tr>
<tr>
<td>SDHD = 2</td>
<td>VHL = 2</td>
</tr>
<tr>
<td>VHL = 2</td>
<td>NF1 = 3</td>
</tr>
<tr>
<td>131I-MIBG scintigraphy positive tumors/total investigated</td>
<td>32/33</td>
</tr>
<tr>
<td>111In-pentreotide scintigraphy positive tumors/total investigated</td>
<td>2/7</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).
sst<sub>2</sub> and D<sub>2</sub> immunohistochemistry analysis

To correlate sst<sub>2</sub> and D<sub>2</sub> 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 sst<sub>2</sub> presence. A prolactin secreting pituitary adenoma was used as positive control of D<sub>2</sub> presence. Immunostaining for sst<sub>2</sub> was performed using polyclonal rabbit antibodies raised against a sequence in the carboxyterminal region of the sst<sub>2A</sub> receptor protein (SS-800; Gramsch Laboratories, Schwabhausen, Germany) diluted at 1:2000. Immunostaining for D<sub>2</sub> was performed using monoclonal mouse antibodies raised against a sequence in the aminoterminal region of the D<sub>2</sub> 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 sst<sub>2</sub>.

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<sub>2</sub> and D<sub>2</sub> mRNA expression in PCC and PGL

All PCC and PGL expressed D<sub>2</sub> mRNA (Table 4 and Fig. 1A). In 72% of tumors, the level of D<sub>2</sub> was higher than 2 copy/copy β-Gus. The D<sub>2</sub> mean mRNA levels were significantly higher in PCC than in PGL (P < 0.002, Table 4). In hereditary tumors, VHL tumors presented the lowest D<sub>2</sub> 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 D<sub>2</sub> 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, sst<sub>2</sub>, and sst<sub>1</sub> were the main subtypes, widely expressed in 100% and 94% of tumors, respectively. Mean sst<sub>2</sub> mRNA level was higher than that of sst<sub>1</sub> mRNA (1.6 ± 1.5 vs 0.6 ± 0.9 copy/copy β-Gus). sst<sub>2</sub> mRNA level was higher than sst<sub>1</sub> 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).

sst<sub>3</sub> was expressed in 53% of PCC/PGL, and sst<sub>5</sub> 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

Table 3 Comparison of immunohistochemistry and mRNA expression levels for sst<sub>2</sub> and D<sub>2</sub> in seven PCC/PGL tumors

<table>
<thead>
<tr>
<th>Tumors</th>
<th>sst&lt;sub&gt;2&lt;/sub&gt; mRNA</th>
<th>Whole</th>
<th>Membrane</th>
<th>Cytoplasmic</th>
<th>D&lt;sub&gt;2&lt;/sub&gt; mRNA</th>
<th>D&lt;sub&gt;2&lt;/sub&gt; 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>+/+ +&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PCC23</td>
<td>2.25</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>9.02</td>
<td>+++</td>
</tr>
<tr>
<td>PCC25</td>
<td>0.79</td>
<td>+</td>
<td>+/+&lt;sup&gt;a&lt;/sup&gt;</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>+/+ +&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PCC29</td>
<td>0.61</td>
<td>+</td>
<td>+/+&lt;sup&gt;a&lt;/sup&gt;</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 medulla</td>
<td>1.16/+/− 0.2</td>
<td>++</td>
<td>++</td>
<td>ND</td>
<td>0</td>
<td>ND</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.

<sup>a</sup>Focal localization.
<sup>b</sup>Heterogenous localization.
In control normal endocrine cells of pancreas, a diffuse positive reaction was found in cytoplasms, 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).

Table 4 Expression mRNA levels of sst1, sst2, sst3, sst5, D2, 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/PGLa</th>
<th>GEP-NETsb</th>
<th>PCC/ GEP-NETsc</th>
<th>PGL/ GEP-NETsc</th>
</tr>
</thead>
<tbody>
<tr>
<td>sst1</td>
<td>0.53±0.13</td>
<td>0.16±0.05</td>
<td>NS</td>
<td>0.07±0.01</td>
<td>P&lt;0.0001</td>
<td>NS</td>
</tr>
<tr>
<td>sst2</td>
<td>1.68±0.23</td>
<td>1.37±0.32</td>
<td>NS</td>
<td>1.53±0.2</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>sst3</td>
<td>0.30±0.01</td>
<td>0.08±0.04</td>
<td>P&lt;0.02</td>
<td>0.09±0.03</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>sst5</td>
<td>0.14±0.09</td>
<td>0.08±0.05</td>
<td>P&lt;0.03</td>
<td>0.78±0.21</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.04</td>
</tr>
<tr>
<td>D2</td>
<td>5.72±0.6</td>
<td>1.92±0.38</td>
<td>P&lt;0.002</td>
<td>0.50±0.21</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>VMAT1</td>
<td>28.7±7</td>
<td>42±11</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VMAT2</td>
<td>35.4±7</td>
<td>41.5±7</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAT</td>
<td>5.9±0.7</td>
<td>2.3±0.4</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 of sst and D2 mRNA levels between PCC/PGL and GEP-NETs

Somatostatin receptor subtypes sst1–3, and sst5 and D2 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 D2 mRNA were significantly higher in PCC and PGL than in GEP-NETs (P<0.0001). Surprisingly, sst2 mRNA levels were similar in both tumor groups (Table 4 and Fig. 2). sst5 mRNA mean levels were more than five times lower in sst5 expressing PCC and PGL than in sst5 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, sst1 and sst3 mRNA mean expression was low and similar in PGL and GEP-NETs, whereas slightly but significantly higher for sst1 in PCC (Table 4). There was no significant difference in sst2 or sst5 mRNA between 111In-pentetreotide-positive (n=4) and -negative tumors (n=12; data not shown).

sst2 immunohistochemistry analysis

Considering the strong unexpected sst2 mRNA expression in PCC/PGL, the presence of sst2 protein was also analyzed also by immunocytochemistry in seven PCC/PGL.

In control normal endocrine cells of pancreas, a diffuse positive reaction was found in cytoplasms, 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).
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<sub>2</sub> receptor protein varied in intensity: lack of immunostaining (<i>n</i> = 1; PCC28), weak immunostaining (<i>n</i> = 3; PGL1, PCC25, and PCC29), moderate immunostaining (<i>n</i> = 2; PCC9 and PGL5), and strong immunostaining (<i>n</i> = 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<sub>2</sub> mRNA was associated with protein expression.

**D<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> protein varied in intensity: lack of immunostaining (<i>n</i> = 1; PGL1), weak/moderate immunostaining (<i>n</i> = 2; PCC9 and PCC28), moderate immunostaining (<i>n</i> = 3; PGL5, PCC25, and PCC29), strong immunostaining (<i>n</i> = 1, PCC23) (Table 3 and Fig. 4). D<sub>2</sub> immunostaining was significantly correlated to the D<sub>2</sub> mRNA expression levels (<i>P</i> < 0.02). However, in PCC9, which presented high D<sub>2</sub> mRNA level, only a weak/moderate D<sub>2</sub> immunostaining was found. This may be partially explained by heterogeneous 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 (<i>P</i> < 0.0001; <i>r</i> = 0.57, global, Fig. 5B; <i>P</i> < 0.0003 for PCC and <i>P</i> < 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 (<i>P</i> < 0.004, Table 4). It was correlated to D<sub>2</sub> mRNA expression in both tumor types (<i>P</i> < 0.0001, <i>r</i> = 0.56, global, Fig. 5C; <i>P</i> < 0.02 for PCC and <i>P</i> < 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, <i>P</i> < 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 <sup>131</sup>I-MIBG scintigraphy-positive (<i>n</i> = 41) and -negative (<i>n</i> = 5) tumors (data not shown).

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**Figure 4** Immunohistochemical analysis of D<sub>2</sub> receptor expression. (A) Granular cytoplasmic and membrane staining in a pituitary prolactin secreting adenoma (1000×). (B) Negative reaction in a case of PGL (PGL1) (400×). (C) Granular cytoplasmic and sometimes membrane staining (arrows) in a case of PCC (PCC25) (400×). Scale bars: 20 μm (B, C); 50 μm (A).
sst2, D2, VMAT1, and VMAT2 mRNA expression in one patient bearing seven PGL or PCC

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

Figure 5 (A) VMAT1, VMAT2, and NAT mRNA expression in 52 tumors, 39 PCC and 13 PGL. 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. (B) Correlation between VMAT1 and VMAT2 mRNA in the same tumors (r=0.57, P<0.0001). (C) Correlation between NAT and D2 mRNA levels in the same tumors (r=0.56, P<0.0001). In (B and C) filled circles = PCC, open circles = PGL.
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, 18F-DOPA, and 18F-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. D2 and VMAT1/2 coexpression in PCC/PGL also raises the question of a possible interaction between D2 and VMAT1/2. A recent study in PC12 PCC cell line showed that a D2 agonist (bromocriptine) displays an inhibitory effect on the 3H 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 D2 agonist (quinpirole) stimulates the uptake of 3H 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 (Temenbaum et al. 1995, van der Harst et al. 2001, Kaltzas 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 sst2 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. 1993, 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 sst2 mRNA are similar to those observed in GEP-NETs, as already suggested by Binderup et al. (2008). However, a lower sst2 expression was observed in four metastatic PCC/PGL by Kolby et al. (2006). The similar mean sst2 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 sst2, 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). sst2 labeling was clearly observed in 6/7 tumors analyzed by immunohistochemistry, confirming the presence of sst2 protein in our series. However, the cytoplasmic localization of the sst2 receptor in some tumors might partially explain the failure of sst2 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 sst2 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 sst5. sst5 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 sst5 subtype in the response to the current SRIF analogs (octreotide and lanreotide), which recognize mostly sst2, but have a slight affinity for sst5 too. Indeed, sst2 and sst5 were reported to heterodimerize (Grant et al. 2008), sst2 and sst5 act additively on GH secretion (Saveanu et al. 2001), and sst5 presence drastically modifies sst2 internalization, maybe affecting cellular desensitization to somatostatin (Sharif et al. 2007). Most previous studies have found similar results for sst5, 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 sst5 expression in all six investigated PCC (Pasquali et al. 2008).

sst1 is constantly expressed in PCC/PGL, at lower levels than sst2, 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 sst1 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 sst3 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 111In-pentetreotide is a first-line detection tool with high sensitivity/specificity (Schmidt et al. 2002, Duet et al. 2003, Koopmans et al. 2008). However, in PCC, its sensitivity is clearly lower than that of 18F-DA PET and MIBG scintigraphy.
A Saveanu, M Muresan et al.: sst and D2 in pheochromocytomas

(van der Harst et al. 2001, Ilias et al. 2008). New 68Ga-labeled octreotide derivatives used for PET imaging have improved the sensitivity in detection of PCC compared with 111In-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 (177Lu)- or Yttrium (90Y)-radiolabeled 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 sst1, sst2, sst3, and sst5, showed better efficacy than octreotide in the suppression of ACTH and GH secretion from pituitary tumor cells (van der Hoek et al. 2009, Petersen 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 sst1 and sst2 found in our series suggests that pasireotide effect may pass through sst1, rather than sst5 or sst3, in this type of tumor. Furthermore, the coexpression of ssts and of D2 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 radiolabeled 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 131I-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 sst2. The authors suggested a complementary role from NAT/VMAT transporters added to a predominant sst2 contribution (Vaidyathanathan et al. 2007).

In conclusion, we demonstrated that PCC and for the first time PGL both express high levels of D2 mRNA, clearly higher than GEP-NETs. Moreover, PCC/PGL co-express somatostatin receptors sst1 and sst2 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 D2 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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