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Differential expression and processing of secretogranin II in relation to the status of pheochromocytoma: implications for the production of the tumoral marker EM66

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Introduction

Granins or chromogranins/secretogranins (Cgs/Sgs) constitute a family of secretory proteins stored in large dense-core vesicles of neurons and neuroendocrine cells (Winkler & Fischer-Colbrie 1992, Fischer-Colbrie *et al.* 1995). Their amino acid sequences are characterized by the abundance of acidic residues and the occurrence of several pairs of consecutive basic residues forming cleavage sites for endopeptidases. Thus, granins serve as precursor proteins that can be processed by prohormone convertases (PCs), allowing production of bioactive peptides (Montero-Hadjadje *et al.* 2008). In particular, the sequence of human secretogranin II (SgII or SCG2 as given in the HUGO database) contains nine pairs of basic amino acids that represent potential

cleavage sites by PCs. Indeed, proteolytic processing of SgII leads to the production of several peptides, such as secretoneurin (SN; SgII₁₅₂₋₁₈₄) which regulates neurotransmission, inflammatory responses, and gonadotrope activity (Fischer-Colbrie *et al.* 2005, Shyu *et al.* 2008, Zhao *et al.* 2010); EM66 (SgII₁₈₇₋₂₅₂) which may participate in the control of feeding behavior in rodents (Boutahricht *et al.* 2005, 2007); and manserin (SgII₄₉₇₋₅₃₆) whose putative role remains to be established (Yajima *et al.* 2004, 2008, Tano *et al.* 2010). The ubiquitous distribution of granins and their derived peptides in nervous, endocrine, and neuroendocrine tissues makes these entities useful markers of secretion from neuroendocrine cells and neoplasms (Rosa & Gerdes 1994). Thus, Cgs/Sgs and Cgs/Sgs-derived peptides may be used for the diagnosis and/or

prognosis of neuroendocrine tumors (Conlon 2010, Portela-Gomes *et al.* 2010). For instance, high concentrations of SgII were found in ganglioneuromas and neuroblastomas (Eder *et al.* 1998), and high plasma SN concentrations were associated with several neuroendocrine tumors (Ischia *et al.* 2000a, Stridsberg *et al.* 2008) or progression of neuroendocrine prostatic carcinomas (Ischia *et al.* 2000b).

Pheochromocytomas are rare catecholamine-producing tumors originating from chromaffin tissues at adrenal and extra-adrenal locations (the latter referred to as paragangliomas). Most of these neuroendocrine tumors occur sporadically, but ~25% result from germline mutations in seven genes identified to date, i.e. the oncogene *RET* or the oncosuppressors von Hippel-Lindau (*VHL*), neurofibromatosis 1 (*NFI*), genes associated with the succinate dehydrogenase complex (*SDHA*, *-B*, *-C*, *-D*, and *SDHAF2*), and also the novel tumor suppressor gene *TMEM127* (Karasek *et al.* 2010). In ~10% of cases, pheochromocytomas can present as or subsequently develop into malignant disease with a poor prognosis (Chrisoulidou *et al.* 2007). The malignant behavior of pheochromocytoma remains poorly understood, and there is a need for improved predictors of malignancy. Unlike benign tumors that can be diagnosed and surgically treated, there is currently no clinical, biochemical, or histological tools that can reliably identify, predict, or cure malignant pheochromocytomas. Malignant tumors can be diagnosed only after metastasis appearance (Kantorovich & Pacak 2010). Recent comparative microRNA and mRNA array studies made it possible to identify a set of genes that could discriminate benign from malignant tumors and to gain insights into the potential mechanisms underlying the occurrence of malignancy in pheochromocytomas (Thouënnon *et al.* 2007, Suh *et al.* 2009, Meyer-Rochow *et al.* 2010, Waldmann *et al.* 2010). In addition, we have demonstrated that EM66 levels increase significantly in the plasma of patients with pheochromocytomas (Guillemot *et al.* 2006) and that low concentrations of this SgII-derived peptide in tumoral tissue are associated with malignancy (Yon *et al.* 2003), indicating that EM66 may represent a valuable new diagnostic and prognostic marker of pheochromocytomas (Guerin *et al.* 2010).

The aim of the present study was to investigate the molecular mechanisms leading to the marked variations in EM66 levels in pheochromocytomas. For this purpose, we compared the gene expression levels of *SgII*, *PCI*, and *PC2*, and investigated the proteolytic processing of SgII using a series of polyclonal antibodies directed against SgII and several derived peptides, in normal adrenal glands, and in benign and malignant pheochromocytomas. Since the regulation of *SgII* expression is dependent on the level of p-cAMP-responsive element-binding (CREB) expression (Scammell *et al.* 2000) and that the absence of p-CREB may be linked to the development of

malignant tumors (Rosenberg *et al.* 2003), we also investigated the p-CREB protein expression in benign and malignant pheochromocytomas.

Materials and methods

Patients and tissue collection

In this study, we analyzed tissues from 13 normal adrenal glands, and 35 benign and 16 malignant pheochromocytomas. The tumors were obtained from 26 women (mean age 46.3 ± 17.1 year, range 15–68 year) and 25 men (46.0 ± 15.9 year, range 13–67 year). Among the tumors, 36 were located in the adrenal and 15 were at an extra-adrenal site (paraganglioma). Thirty-eight tumors showed no evidence of hereditary disease, i.e. sporadic, whereas four showed an *SDHB* mutation, one an *SDHD* mutation, one a *VHL* mutation, four a *RET* mutation, and three an *NFI* mutation. The tissues were provided by a French endocrinology network for collection of adrenal tumors (Réseau COMETE-3, PHRC AOM-06179) and by the Rouen and the Nancy University Hospital Centers. Tumor specimens were collected during surgery and immediately frozen on dry ice. After collection, tissue samples were kept frozen at -80°C . Malignancy was established on the basis of the presence of at least one metastasis. Normal adrenal tissues were obtained either from brain-dead organ donors or from patients undergoing nephrectomy for kidney cancer. The protocols of collection of the samples and the experimental procedures were approved by the regional bioethics committee, and informed consent was obtained from all the patients with or without pheochromocytomas.

Antibodies

Antibodies against SgII (VV493-P, hSgII₉₋₅₅₂), N-term (N161-S, hSgII₉₋₁₅₂), Int (I180-C, hSgII₂₆₇₋₄₄₆), C-Term (C107-B, hSgII₄₄₆₋₅₅₂), EM66 (code no. 736-1806, hSgII₁₈₇₋₂₅₂), SN (code no. 733-145, hSgII₁₅₂₋₁₈₄), PCI (code no. 9212-07, PCI₆₂₉₋₇₂₆), and PC2 (code no. 7551-05, PC2₅₂₉₋₆₃₇) were raised in rabbit (Benjannet *et al.* 1993, Vallet *et al.* 1995, 1997, Anouar *et al.* 1996). The mouse monoclonal antibody against actin and the rabbit polyclonal antibody against p-CREB1 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The mouse monoclonal antibody against α -tubulin was obtained from Sigma-Aldrich St. Louis, MO.

Tissue extraction

For EM66 RIA, frozen tissue samples were boiled for 10 min in 0.5 M acetic acid, homogenized in a glass Potter, sonicated, and centrifuged (3000 g, 4 °C) for 30 min. The supernatants were collected and kept at

4 °C until prepurification. The pellets were used for the measurement of protein concentrations by the Bradford method.

For Western blot analysis, pheochromocytoma tissues were homogenized in 50 mM Tris—HCl (pH 7.4), containing 1% Triton X-100, 10 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation (21 000 g, 4 °C) for 15 min, the proteins contained in the supernatant were quantified using the Bradford method. Proteins were then precipitated by trichloroacetic acid (10% final concentration). Due to the scarcity of the tumor samples and use of the tissues for the RIA and quantitative reverse transcription PCR (Q-RT-PCR) experiments, Western blots were only performed from five benign and four malignant pheochromocytoma extracts.

Prepurification of tissue extracts

Each sample was loaded onto a Sep-Pak C₁₈ cartridge (Waters Corp., St-Quentin en Yvelines, France) equilibrated with a solution of 0.1% trifluoroacetic acid (TFA) in water. Bound material was eluted from the cartridge with a solution of acetonitrile/water/TFA (59:9:40:0.1, vol/vol/vol), dried by vacuum centrifugation and kept at room temperature until RIA. EM66 quantification was performed as described previously (Anouar *et al.* 1998, Guillemot *et al.* 2006).

Quantitative reverse transcription PCR

Total RNA was extracted from the normal adrenal gland and pheochromocytoma specimen by the guanidinium–thiocyanate–phenol–chloroform method (Chomczynski & Sacchi 1987) using Trizol reagent (Sigma–Aldrich). Contaminating DNA was removed by treatment with RNase-free DNase I (Promega), and cDNA was synthesized with the ImProm II Reverse Transcription System for RT-PCR (Promega) by reverse transcription of 0.4 µg of total RNA. Q-RT-PCR was performed on cDNA in the presence of a 1× SYBR Green Master Mix (Applied Biosystems, Courtaboeuf, France) containing pre-set concentrations of dNTPs, MgCl₂, and the buffer, along with 300 nM forward and reverse primers, using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). mRNA levels were determined by comparison of the signals generated from cDNA in samples with those generated by a standard curve constructed with known amounts of cDNA, and internally corrected with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA signal for variations in the amounts of input mRNA. The primers used were as follows: *SgII* forward primer 5'-agccgaatggatcagtgaa-3' and reverse primer 5'-gatgtctaagtcagcctctgagat-3'; *PCI* forward primer 5'-ggatgatggtttggagtgaa-3' and reverse primer 5'-gggaaatgatcatggtcattatc-3'; *PC2* forward

primer 5'-agggaaaggtgttaccattgga-3' and reverse primer 5'-tgtgtaccgagggttaaggtagg-3'; *GAPDH* forward primer 5'-gagccaaaagggtcatcatc-3' and reverse primer 5'-ccatc-cacagtctctgggt-3'.

Western blot analysis

Proteins were analyzed by PAGE under denaturing conditions (SDS–PAGE, 10% total acrylamide), electroblotted onto nitrocellulose membranes (Amersham Pharmacia Biotech, Orsay, France) and revealed with the various antisera using a chemiluminescence detection kit (Amersham Pharmacia Biotech). Antisera directed against EM66 and SN were used at a dilution of 1:4000 and 1:1000 respectively. SgII antibodies were used at a dilution of 1:600 and anti-N-term, -Int, and -C-Term at a dilution of 1:500. Antisera against PC1 and PC2 were both used at a dilution of 1:1000. Antibodies against p-CREB1 were used at a dilution of 1:100. For all samples, 20 µg of protein extract was used to allow semi-quantitative comparison of the immunoreactivity. To correct for protein loading variation, the blots were normalized using the structural protein actin detected with a monoclonal actin antiserum at a dilution of 1:150 or by the α-tubulin antiserum at a dilution of 1:2500. Bound primary antibodies were detected using anti-rabbit or anti-mouse IgG-HRP secondary antibodies. Immunoblotting was done on a total of five benign and four malignant pheochromocytomas and was repeated three times. Autoradiographic films were quantified using an image analysis system (Samba, Grenoble, France).

Statistical analysis

Data are reported as median (25th–75th percentile), where the 25th–75th percentile represents the dispersion of the distribution. Statistical analysis of the data was performed by the Mann–Whitney *U* test. The diagnostic performance of the EM66 test was evaluated by receiver-operating characteristic (ROC) curve analysis. The correlation between variables was evaluated by Spearman's nonparametric test. Probability values <0.05 were considered significant. Data were analyzed and graphics generated with the Prism program (GraphPad Software, Inc., San Diego, CA, USA).

Results

EM66 levels in pheochromocytoma extracts

In patients with benign pheochromocytomas (*n*=24), EM66 concentrations ranged from 3.2 to 1221 ng/mg protein with a median value of 103.4 ng/mg protein. The concentration of EM66 in malignant tumors (*n*=10) ranged from 2.9 to 9.7 ng/mg protein with a median value of 6.4 ng/mg protein. Statistical analysis revealed that the concentration of EM66 was

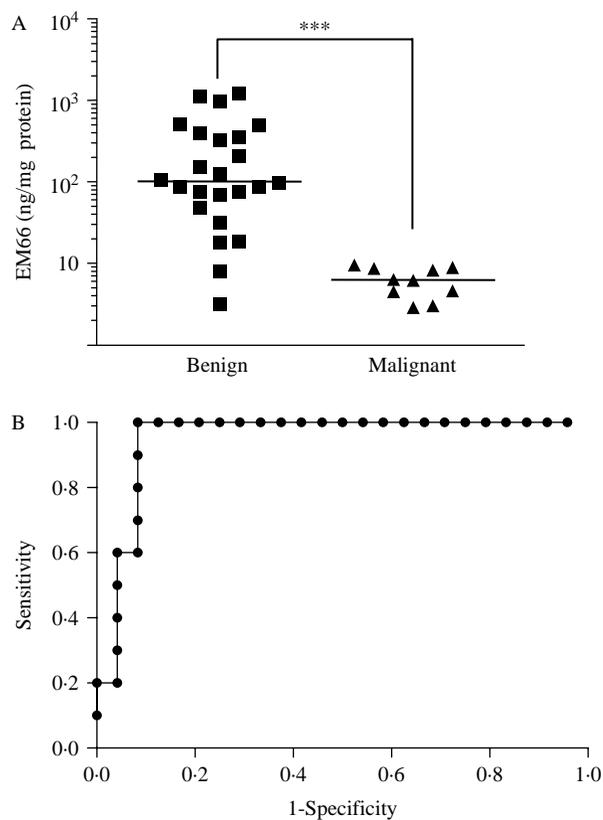


Figure 1 (A) Scattergram of EM66 concentrations in benign (closed square) and malignant (closed triangle) pheochromocytoma extracts as determined by RIA quantification. The bars represent the median value for each group. *** $P < 0.001$. (B) ROC curve showing diagnostic performance of EM66 in patients with benign or malignant pheochromocytoma. Each point represents ROC curve relating changes in true-positive results (sensitivity) and false-positive results (1-specificity) for predicting malignancy as a function of changing cut-offs for EM66 concentrations.

significantly higher in benign than in malignant tumors ($P < 0.001$; Fig. 1A). The performance of the EM66 test for the diagnosis of malignant pheochromocytoma was determined on the basis of a cut-off value of 14 ng/mg protein, based on the ROC curve analysis. We calculated a sensitivity of 100%, a specificity of 92%, a positive predictive value of 83%, a negative predictive value of 100%, and an area under the ROC curve (AUC; 95% confidence interval) of 0.95 (0.88–1.02) (Fig. 1B). No significant correlation was found between EM66 levels and tumor size (Spearman's correlation coefficient = -0.04 ; $P = 0.816$; data not shown).

***Sgll* mRNA and protein levels in normal adrenal glands and pheochromocytomas**

Using Q-RT-PCR, we determined that in normal adrenal glands, and benign and malignant pheochromocytomas, *Sgll* mRNA levels were 14.03 (4.72–41.07);

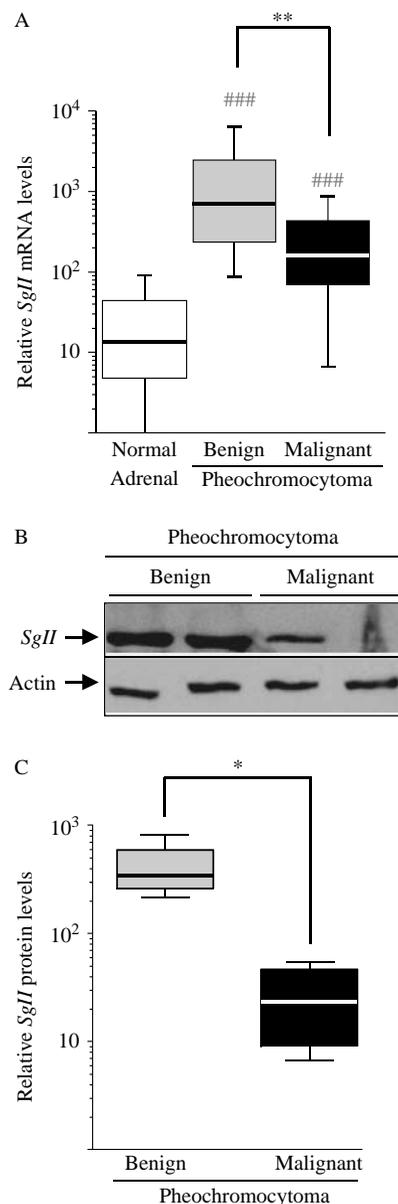


Figure 2 Expression of *Sgll* in normal adrenal glands, and benign and malignant pheochromocytomas. (A) Comparative quantification of *Sgll* mRNA expression in normal adrenal glands, and benign and malignant pheochromocytomas. mRNA levels were determined by quantitative real-time PCR, as described in Materials and methods. (B) Representative Western blot of *Sgll* in benign and malignant pheochromocytoma extracts. Immunoreactive bands of ~97 and ~46 kDa corresponding to the human *Sgll* protein and actin respectively are detected. Actin was used as an internal control. (C) Relative amounts of *Sgll* protein in five benign vs four malignant chromaffin tumors. The box represents the interquartile range (25–75th percentile) and the line within the box corresponds to the median value. The bottom and top bars of the whisker indicate the minimum and maximum values respectively. ### $P < 0.001$, pheochromocytomas vs normal adrenal glands; * $P < 0.05$; ** $P < 0.01$, benign vs malignant pheochromocytomas.

661.7 (227.2–2271), and 145 (65.41–390.8) respectively (Fig. 2A). *SgII* mRNA levels were significantly lower in normal adrenal glands than in benign or malignant tumors ($P < 0.001$). Moreover, *SgII* expression in benign pheochromocytomas was about fivefold higher than in malignant neoplasms ($P < 0.01$; Fig. 2A). We also observed that *SgII* mRNA levels measured by Q-RT-PCR in pheochromocytomas are in good correlation with the result observed in our microarray study (Thouennon *et al.* (2007); Spearman's correlation coefficient = 0.58; $P < 0.05$; data not shown). *SgII* mRNA and EM66 peptide levels were found to be significantly correlated (Spearman's correlation coefficient = 0.66; $P < 0.001$; data not shown), whereas *SgII* mRNA levels and tumor size were not (Spearman's correlation coefficient = 0.04; $P = 0.831$; data not shown).

In benign and malignant pheochromocytoma samples examined by western blot, the SgII antibodies revealed the presence of a band with an apparent molecular mass of ~97 kDa corresponding to the size of the human SgII protein, as reported previously (Vallet *et al.* 1997; Fig. 2B). For semi-quantification of the immunoblots, SgII levels in pheochromocytoma extracts were normalized to the actin protein band probed simultaneously in each sample (Fig. 2B). We found significantly higher concentrations of SgII in benign tumors compared with malignant tumors (345.9 (303.9–413.3) % vs 23.7 (14.2–36.2) %, $P < 0.05$; Fig. 2C).

PC1 and PC2 mRNA and protein levels in normal adrenal glands and pheochromocytomas

PC1 mRNA levels in normal adrenal glands, and benign and malignant pheochromocytomas were respectively 7.825 (3.985–22.8), 247.3 (153.6–355.4), and 65.8 (33.62–133.6) (Fig. 3A). *PC2* mRNA levels in normal adrenal glands, and benign and malignant pheochromocytomas were respectively 9.015 (4.235–24.07), 278.4 (165.2–570.8), and 90.51 (40.22–154.4) (Fig. 4A). As observed for *SgII*, *PC1* and *PC2* expression levels in normal adrenal glands were significantly lower compared with benign or malignant tumors ($P < 0.001$). In addition, in benign pheochromocytomas, *PC1* and *PC2* expression was about three to four times higher than in malignant tumors ($P < 0.01$ and $P < 0.001$ respectively; Figs 3A and 4A). As for *SgII* mRNA, *PC1* and *PC2* mRNA levels measured by Q-RT-PCR in pheochromocytomas are in good correlation with the result of our previous microarray study (Thouennon *et al.* (2007); Spearman's correlation coefficient = 0.48, $P < 0.05$ for *PC1*; Spearman's correlation coefficient = 0.52, $P < 0.05$ for *PC2*; data not shown).

Western blot experiments using the PC1 antibodies detected three bands of ~89, ~83, and ~66 kDa

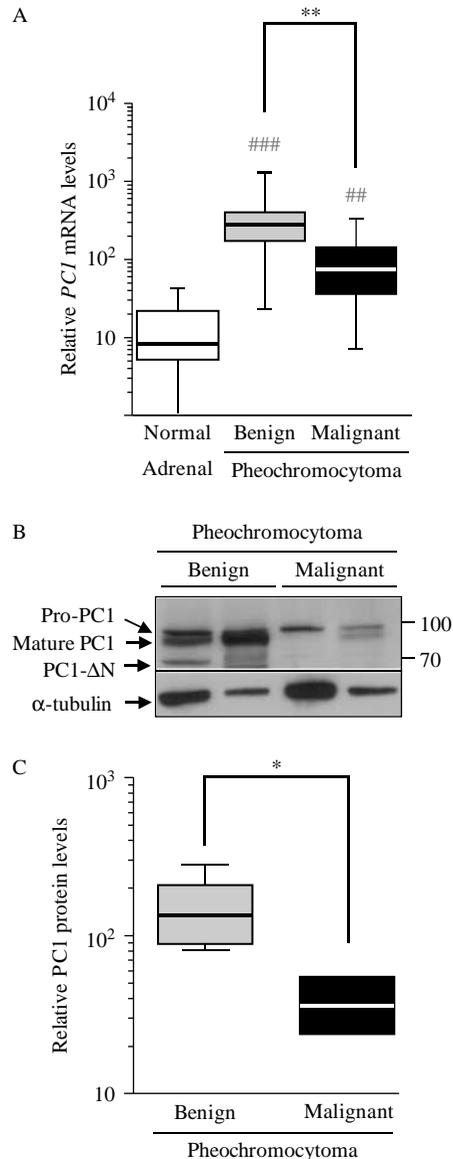


Figure 3 Expression of PC1 in normal adrenal glands, and benign and malignant pheochromocytomas. (A) Comparative quantification of *PC1* mRNA expression in normal adrenal glands, and benign and malignant pheochromocytomas. mRNA levels were determined by quantitative real-time PCR, as described in Materials and methods. (B) Representative western blot of PC1 in benign and malignant pheochromocytoma extracts. Immunoreactive bands of ~89, ~83 and ~66 kDa corresponding to the precursor (pro-PC1), the active form (mature PC1), and the N-terminally truncated form (PC1-ΔN) of the PC1 protein respectively are detected. α-Tubulin was used as an internal control. Molecular weight markers (in kDa) are indicated. (C) Relative amounts of the PC1 (89+83+66 kDa forms) protein in five benign vs four malignant chromaffin tumors. The box represents the interquartile range (25th–75th percentile) and the line within the box corresponds to the median value. The bottom and top bars of the whisker indicate the minimum and maximum values respectively. ### $P < 0.01$; ### $P < 0.001$, pheochromocytomas vs normal adrenal glands; * $P < 0.05$; ** $P < 0.01$, benign vs malignant pheochromocytomas.

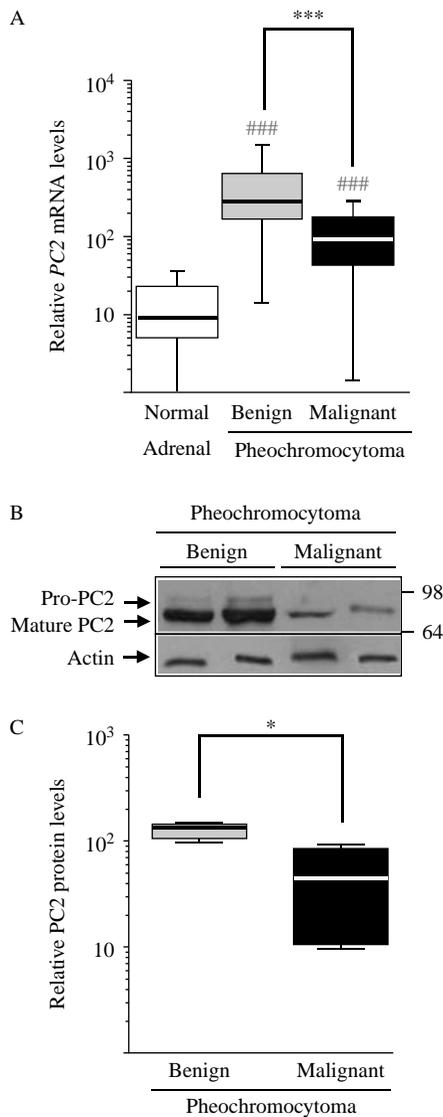


Figure 4 Expression of PC2 in normal adrenal glands, and benign and malignant pheochromocytomas. (A) Comparative quantification of PC2 mRNA expression in normal adrenal glands, and benign and malignant pheochromocytomas. mRNA levels were determined by quantitative real-time PCR, as described in Materials and methods. (B) Representative western blot of PC2 in benign and malignant pheochromocytoma extracts. Immunoreactive bands of ~75 and ~66 kDa corresponding to the precursor (pro-PC2) and active (mature PC2) form of the PC2 protein respectively are detected. Actin was used as an internal control. Molecular weight markers (in kDa) are indicated. (C) Relative amounts of the PC2 (75+66 kDa forms) protein in five benign vs four malignant chromaffin tumors. The box represents the interquartile range (25th–75th percentile) and the line within the box corresponds to the median value. The bottom and top bars of the whisker indicate the minimum and maximum values respectively. ### $P < 0.001$, pheochromocytomas vs normal adrenal glands; * $P < 0.05$; *** $P < 0.001$, benign vs malignant pheochromocytomas.

corresponding respectively to the precursor (pro-PC1), to the active form (mature PC1), and to the N-terminally truncated form (PC1-ΔN) of the enzyme (Fig. 3B). Semi-quantification of the immunoblots revealed that the amount of PC1 (89+83+66 kDa forms) was significantly higher in benign than in malignant tumors (131 (91.8–209.3) % vs 40 (24–56) %, $P < 0.05$) (Fig. 3C). The antiserum directed against PC2 revealed two bands of ~75 and ~66 kDa corresponding respectively to the precursor (pro-PC2) and to the active form (mature PC2) of the enzyme (Fig. 4B). Semi-quantification of the immunoblots revealed that the amount of PC2 (75+66 kDa forms) was significantly higher in benign than in malignant tumors (118 (97.6–124.3) % vs 42.5 (10.4–81.1) %, $P < 0.05$) (Fig. 4C).

Maturation of SgII in normal adrenal glands and pheochromocytomas

Using antisera directed against sequential regions of the SgII protein, i.e. N-terminal, SN, EM66, internal, and C-terminal (Fig. 5A), we observed by western blot distinct processing profiles between normal adrenal glands and pheochromocytomas, on the one hand, and between benign and malignant chromaffin tumors, on the other hand (Fig. 5B). The semi-quantitative analysis of the immunoreactive signals for each tissue sample is summarized in Table 1. Protein bands of ~97 (SgII), ~80, ~64, and ~46 kDa were mainly observed in normal adrenal glands, while other bands including ~31 and ~15 kDa bands were exclusively detected in tumoral tissue samples. Whatever the antiserum used, benign pheochromocytomas showed higher quantities and more numerous immunoreactive bands compared with malignant tumors. Only the C-term antibodies allowed the detection of an ~22 kDa band in all tissue extracts while an ~15 kDa band was observed with the SgII antisera exclusively in benign pheochromocytomas.

CREB protein levels in pheochromocytomas

In benign and malignant pheochromocytomas, western blot experiments using an antiserum directed against the p-CREB protein revealed the presence of a band corresponding to the mature protein (Fig. 6A). Semi-quantification of the immunoreactive signals showed higher levels of p-CREB in benign pheochromocytomas compared with malignant pheochromocytomas (251 (16.7–616.8) % vs 30.7 (2.9–63) %). Although p-CREB levels were not statistically different between the two groups of tumors (Fig. 6B), a significant correlation was found between the p-CREB protein and SgII mRNA levels (Spearman's correlation coefficient=0.81; $P < 0.05$; Fig. 6C).

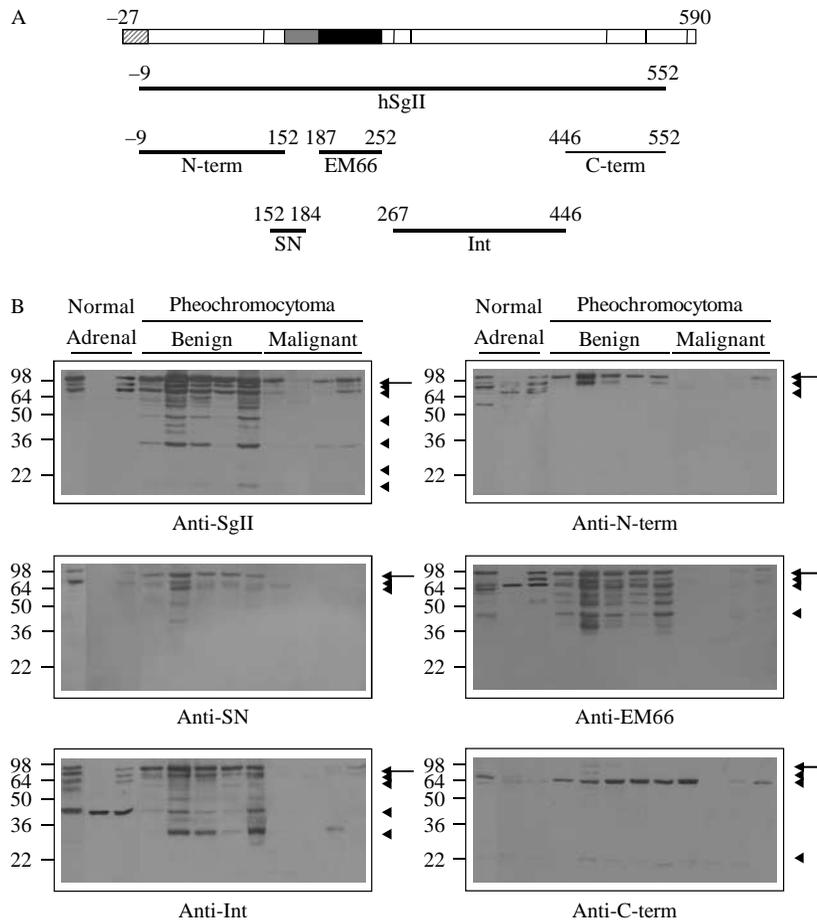


Figure 5 (A) Schematic representation of human SgII. The regions of the protein (with the position of the N- and C-terminal amino acids) against which polyclonal antisera have been produced are indicated. Hatched square represents the signal peptide; gray square, secretoneurin; black square, EM66. hSgII, full-length human SgII; N-term, hSgII₉₋₁₅₂ N-terminal peptide; SN, secretoneurin; Int, hSgII₂₆₇₋₄₄₆ internal peptide; C-term, hSgII₄₄₆₋₅₅₂ C-terminal peptide. (B) Western blot analysis of normal adrenal glands, and benign and malignant pheochromocytoma extracts using the different antisera described above. Arrow indicates the ~97 kDa apparent molecular weight of hSgII, and arrowheads, the related peptides of ~80, ~64, ~46, ~31, ~22, and ~15 kDa. Molecular weight markers (in kDa) are also indicated.

Discussion

Most of the pheochromocytomas are benign tumors but about 10% of these neoplasms are malignant. To date, the only evidence for malignancy is the occurrence of metastases, which are associated with a poor survival rate. Several studies have evaluated different parameters to predict malignancy of pheochromocytomas (Chrisoulidou *et al.* 2007), and different markers that may help to distinguish benign from malignant tumors have been identified but unfortunately none gives an absolute distinction. In this context, we have previously reported that a low EM66 concentration in

pheochromocytoma tissue is indicative of a malignant status of the tumor (Yon *et al.* 2003). The present study substantiates our preliminary observations. Indeed, we measured the concentrations of EM66 in 24 benign and ten malignant pheochromocytomas, and found that EM66 levels are 16-fold higher in benign than in malignant tumors. A cut-off value of 14 ng EM66/mg protein determined a 100% sensitivity, a 92% specificity, and an AUC of 0.95 for distinguishing benign from malignant tumors. In the literature, only a few studies evaluated the diagnostic accuracy of the markers used in discriminating malignant from benign pheochromocytomas (Gao *et al.* 2006, Suh *et al.* 2009,

Table 1 Relative abundance of the SgII-derived peptides identified by western blot

	Antibodies					
	SgII	N-Ter	SN	EM66	Int	C-term
MW (kDa)						
Normal adrenal gland						
97	+++	++	++	+++	+++	-
80	++	++	++	+++	++	++
64	+++	++	-	+++	+++	+
46	-	-	-	+	+++	-
31	-	-	-	-	-	-
22	-	-	-	-	-	+
15	-	-	-	-	-	-
Benign pheochromocytoma						
97	+++	+++	+++	+++	+++	+
80	+++	+++	++	++	++	+
64	+++	-	++	+++	-	+++
46	++	-	-	++	++	-
31	+++	-	-	-	+++	-
22	+	-	-	-	-	+
15	+	-	-	-	-	-
Malignant pheochromocytoma						
97	++	+	-	+	+	-
80	+	-	-	+	+	-
64	+	-	-	+	-	++
46	-	-	-	+	-	-
31	+	-	-	-	+	-
22	-	-	-	-	-	+
15	-	-	-	-	-	-

+++ , Strong signal; ++ , moderate signal; + , weak signal; - , no signal; MW , molecular weight.

Eisenhofer *et al.* 2011). Moreover, while granin-derived peptides have been reported to be produced in pheochromocytoma (e.g. pancreastatin, chromacin, WE14, EL35 from CGA; GAWK from CGB; SN from SgII), they rarely distinguished the two types of pheochromocytoma (for reviews, see Conlon (2010), Guerin *et al.* (2010) and Portela-Gomes *et al.* (2010)). Our study indicates that EM66 measurement discriminates between benign and malignant tumors with a high accuracy. The AUC of 0.95 is in the same range as that of the pheochromocytoma of the adrenal gland scaled score (PASS system; Thompson (2002)) calculated by Gao *et al.* (2006) and that of the expression of a panel of five genes determined by Suh *et al.* (2009). In addition, the recent work by Eisenhofer *et al.* (2011) on a large population of pheochromocytoma patients indicates that plasma methoxytyramine constitutes a novel marker of malignancy (AUC=0.739). However, the use of the PASS remains controversial (Wu *et al.* 2009, Agarwal *et al.* 2010). Moreover, microarray studies (Thouennon *et al.* 2007, Waldmann *et al.* 2010) did not confirm that the five candidate genes identified by Suh *et al.* (2009) were differentially expressed between different sets of benign and malignant tumors. In this context, despite the 92% specificity, the EM66 test appears very promising and indicates that the peptide

constitutes a marker of malignancy. In addition, apparent benign pheochromocytomas may turn out to be malignant in further follow-up (Timmers *et al.* 2008), which suggests that patients with low tumoral EM66 levels (<14 ng/mg protein) should undergo a careful survey.

The mechanisms leading to low EM66 levels in malignant pheochromocytomas remain obscure. Numerous studies have characterized EM66 as well as SgII mRNA and protein levels in human normal adrenal glands and in pheochromocytomas (Schober *et al.* 1987, Portela-Gomes *et al.* 2004, Guerin *et al.* 2010). Our data from Q-RT-PCR showed that SgII mRNA was over-expressed in tumoral chromaffin tissue compared with normal chromaffin tissue. These results indicate that tumorigenesis of chromaffin cells is accompanied by an increased expression of SgII mRNA, and support the notion that SgII is a useful diagnostic marker for neuroendocrine tumors (Schober *et al.* 1987, Vallet *et al.* 1997). Moreover, in correlation with our microarray study (Thouennon *et al.* 2007), we found that SgII mRNA levels are lower in malignant pheochromocytomas compared with benign pheochromocytomas. We hypothesized that these differences in SgII gene expression could be related to the level of CREB activation in pheochromocytoma. Previous studies have

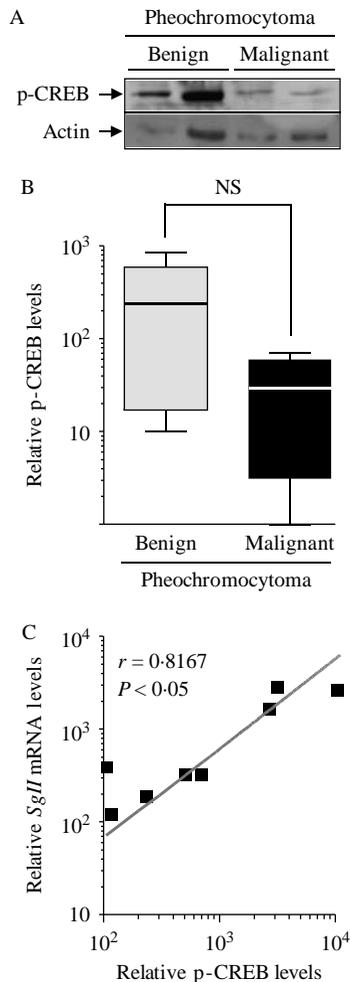


Figure 6 Expression of p-CREB in benign and malignant pheochromocytomas. (A) Representative western blot of p-CREB in benign and malignant pheochromocytoma extracts. Actin was used as an internal control. (B) Relative amounts of p-CREB in five benign vs four malignant chromaffin tumors. The box represents the interquartile range (25th–75th percentile) and the line within the box corresponds to the median value. The bottom and top bars of the whisker indicate the minimum and maximum values respectively. (C) Correlation between *SgII* mRNA expression measured by Q-RT-PCR and p-CREB protein levels in five benign and four malignant pheochromocytomas (closed square) r , Spearman's correlation coefficient. NS, not significant.

shown that *SgII* gene transcription is dependent on the levels of p-CREB expression (Scammell *et al.* 2000) and that neuropeptides and hormones stimulate *SgII* gene expression via CRE-binding proteins such as p-CREB (Turquier *et al.* 2001, Song *et al.* 2003). The results revealed lower p-CREB protein levels in malignant tumors, and a strong correlation between *SgII* mRNA and p-CREB levels. Interestingly, this tendency of p-CREB to decrease in malignant pheochromocytomas is in line with data showing a decrease in p-CREB

protein levels in adrenocortical carcinomas compared with adrenocortical adenomas (Rosenberg *et al.* 2003). Our data suggest that low levels of p-CREB could account for decreased *SgII* expression in malignant pheochromocytomas. Thus, in pheochromocytomas, as in neuroblastomas (Pagani *et al.* 1992), *SgII* mRNA levels correlate with the differentiation status of tumoral cells (benign vs malignant), suggesting that measurement of the *SgII* transcript is a potential predictor of malignancy.

Western blot analysis indicated that SgII protein and SgII-derived fragments were less expressed in malignant than in benign neoplasms. This study and our previous studies demonstrated the usefulness of the SgII-derived peptide EM66 as a diagnostic and prognostic marker of pheochromocytomas (Yon *et al.* 2003, Guillemot *et al.* 2006). The correlation between the *SgII* transcript and EM66 levels indicates that low EM66 concentrations in malignant pheochromocytomas are attributable to the decrease in SgII biosynthesis in these tumors. Finally, it is important to note that gene expression profiling studies of benign and malignant pheochromocytomas revealed underexpression of more than 80% of the differentially expressed genes in malignant tumors (Thouënon *et al.* 2007, Waldmann *et al.* 2010), further emphasizing a potential general decrease in neuroendocrine traits in malignant tumors compared with benign tumors. Such a decrease could be ascribed to altered expression and activation of upstream regulators such as CREB which is known to play a major role in defining the neuroendocrine phenotype.

To further elucidate the mechanisms of differential processing of SgII and production of the EM66 marker, we investigated the proteolytic cleavage of the precursor protein in pheochromocytomas by western blot, using antisera against different SgII-derived fragments and against the prohormone convertases PC1 and PC2. As observed with the SgII antiserum, we found less immunoreactive bands and lower SgII protein and SgII-derived fragments in malignant tumors compared with benign tumors using antibodies recognizing different regions of the protein. Several studies indicated that SgII processing may vary depending on the tissue or cell type (Dittie & Tooze 1995, Muller & Tougaard 1995, Vallet *et al.* 1997, Van Horssen & Martens 1999, Peinado *et al.* 2006, Zhao *et al.* 2006). In this study, we showed that two fragments of ~46 and ~31 kDa were abundant in benign pheochromocytomas. Interestingly, these two fragments were also described in human pituitary gland tumors (Vallet *et al.* 1997). The absence of the ~31 kDa fragment in normal chromaffin cells suggests that SgII undergoes differential processing in normal vs tumoral cells. Based on the different antibodies used in this study, and the potential mono/dibasic cleavage sites in the human *SgII*

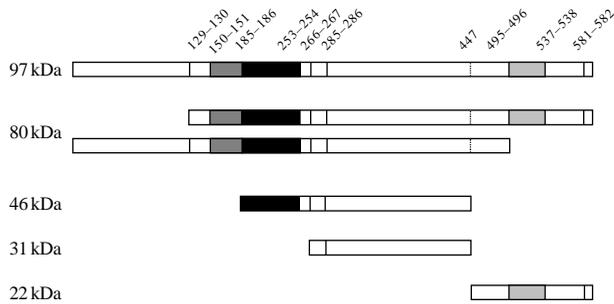


Figure 7 Schematic representation of human SgII processing in pheochromocytomas deduced from the analysis of the immunoblots generated by the antisera directed against different regions of the granin protein. The potential dibasic (solid lines) and monobasic (dashed line) cleavage sites of SgII are indicated. Dark gray, black and light gray squares show the location of SN, EM66, and manserin respectively. The apparent molecular weights of SgII-derived fragments are indicated.

sequence, we propose a possible pattern of processing of SgII in pheochromocytoma, which is depicted in Fig. 7. In this figure, the ~46 and ~31 kDa fragments may correspond to the internal region of SgII (SgII₁₈₇₋₄₄₆ and SgII₂₆₈₋₄₄₆ respectively), which is cleaved to yield EM66. The ~22 kDa fragment may correspond to the C-terminal region of the protein (SgII₄₄₈₋₅₉₀) that encompasses the manserin peptide. The fact that SgII is proteolytically processed into several peptides in normal and tumoral chromaffin cells reinforces the idea that this protein may function as a prohormone precursor. The role of the different SgII-derived peptides generated deserves further investigation. These peptides are known to be produced through the action of the prohormone convertases PC1 and PC2 (Dittie & Tooze 1995, Hoflehner *et al.* 1995, Laslop *et al.* 1998), which, like granins, may constitute markers of neuroendocrine tumors (Scopsi *et al.* 1995, Portela-Gomes *et al.* 2010). Several studies have reported the expression of PC1 and PC2 in normal adrenal glands and pheochromocytomas by northern blot, RT-PCR, and immunohistochemistry (Konoshita *et al.* 1994, Scopsi *et al.* 1995). In the same manner, using the Q-RT-PCR methodology, our data indicate that PC1 and PC2 are expressed in normal and tumoral chromaffin tissues. Moreover, we observed that *PC1* and *PC2* mRNA levels are higher in pheochromocytomas compared with normal chromaffin tissue, while they are lower in malignant tumors compared with benign tumors. As for *SgII* mRNA, these results correlated with those of our microarray study (Thouennon *et al.* 2007). Western blot experiments showed that PC1 and PC2 protein levels are also lower in malignant vs benign tumors. These results suggest that PC processing activity is altered with malignancy and that low EM66 levels in malignant pheochromocytomas may be partly ascribed to reduced PC levels.

Our data indicate that *SgII* as well as *PC1* and *PC2* mRNA are up-regulated in tumoral chromaffin tissues compared with normal chromaffin tissues and down-regulated in malignant pheochromocytoma compared with benign pheochromocytoma. Malignancy is associated with reduced levels of SgII and SgII-derived peptides, in line with the low concentrations of EM66 found in malignant tumors. In accordance with our studies (Thouennon *et al.* 2007, 2009, 2010) and with the recent data of Waldmann *et al.* (2010), this study strengthens the notion that malignancy is accompanied by a reduction in the expression of various traits of the neuroendocrine phenotype. Consequently, low EM66 concentrations measured in malignant pheochromocytomas could be essentially ascribed to a decrease in SgII biosynthesis probably linked to p-CREB down-regulation. Our study highlights the usefulness of SgII-derived peptides, such as EM66, as new valuable prognostic markers of pheochromocytomas and possibly other neuroendocrine tumors. Finally, *in vitro* experiments performed on tumor cell lines suggest that CgA may affect tissue remodeling and tumor growth (Colombo *et al.* 2002). Moreover, using a mouse pheochromocytoma cell line and syngeneic tumor model, Papewalis *et al.* (2011) recently demonstrated that immunogenic CgA peptides may serve as tumor antigens for novel immunotherapies targeting malignant pheochromocytoma. As mentioned by the authors, and in line with our results, characterization of patterns in the expression of peptide fragments in benign and malignant tumors compared with normal tissue should be useful for identifying the most promising tumor epitopes for immunotherapies targeting secretory granule proteins in future clinical trials. Therefore, in addition to their potential use as predictors for diagnosis and prognosis, granin-derived peptides, such as EM66, could also be considered for use in therapeutic strategies for the treatment of neuroendocrine tumors.

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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