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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Abstract

We have previously demonstrated that measurement of tissue concentrations of the secretogranin II (SgII or SCG2 as listed in the HUGO database)-derived peptide EM66 may help to discriminate between benign and malignant pheochromocytomas and that EM66 represents a sensitive plasma marker of pheochromocytomas. Here, we investigated the gene expression and protein production of SgII in 13 normal adrenal glands, and 35 benign and 16 malignant pheochromocytomas with the goal to examine the molecular mechanisms leading to the marked variations in the expression of EM66 in tumoral chromaffin tissue. EM66 peptide levels were 16-fold higher in benign than in malignant pheochromocytomas and had an area under the receiver-operating characteristic curve of 0.95 for the distinction of benign and malignant tumors. Q-PCR experiments indicated that the SgII gene was significantly underexpressed in malignant tumors compared with benign tumors. Western blot analysis using antisera directed against SgII and SgII-derived fragments revealed lower SgII protein and SgII-processing products in malignant tumors. Western blot also showed that low p-cAMP-responsive element-binding (CREB) concentrations seemed to be associated with the malignant status. In addition, the prohormone convertase PC1 and PC2 genes and proteins were overexpressed in benign pheochromocytomas compared with malignant pheochromocytomas. Low concentrations of EM66 found in malignant tumors are associated with reduced expression and production of SgII and SgII-derived peptides that could be ascribed to a decrease in SgII gene transcription, probably linked to p-CREB down-regulation, and to lower PC levels. These findings highlight the mechanisms leading to lower concentrations of EM66 in malignant pheochromocytoma and strengthen the notion that this peptide is a suitable marker of this neuroendocrine tumor.

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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 (NF1), genes associated with the succinate dehydrogenase complex (SDHA, -B, -C, -D, and SDHA2), 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 (Chisoulidou 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 (Thouemmon 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 (Von 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, PC1, 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 NF1 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, hSgII 9–552), N-term (N161-S, hSgII 9–152), Int (I180-C, hSgII 267–446), C-Term (C107-B, hSgII 146–552), EM66 (code no. 736-1806, hSgII 187–252), SN (code no. 733-145, hSgII 152–184), PC1 (code no. 9212-07, PC1 629–726), and PC2 (code no. 7551-05, PC2 529–637) 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 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 C_{18} 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 (59:40:1, 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'-agggaaaggtgtcattcgggtaa-3' and reverse primer 5'-tggtacaggtgaagcttgcaggtt-3'; GAPDH forward primer 5'- gagcggagggatctcattc-3' and reverse primer 5'-ecacagcatctttggtggt-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...
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).

**SgII 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, SgII mRNA levels were 14.03 (4.72–41.07);
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<0.001). Moreover, in 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-NA) 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.

**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 (4.235–22.07), 278.4 (153.6–355.4), and 65.8 (33-62–133-6) (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.
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 antisem 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 antisem 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 antisem exclusively in benign pheochromocytomas.

CREB protein levels in pheochromocytomas

In benign and malignant pheochromocytomas, western blot experiments using an antisem 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 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.
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,

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**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.
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 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.

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

<table>
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<tr>
<th>Antibodies</th>
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Benign pheochromocytoma

| MW (kDa)   |      |       |    |      |     |        |
| 97         | + + + | + + + | + + | + + + | + + | +      |
| 80         | + + + | + + + | + + | + + + | + + | +      |
| 64         | + + + | + + + | + + | + + + | + + | +      |
| 46         | + + + | + + + | + + | + + + | + + | +      |
| 31         | + + + | + + + | + + | + + + | + + | +      |
| 22         | + + + | + + + | + + | + + + | + + | +      |
| 15         | + + + | + + + | + + | + + + | + + | +      |

Malignant pheochromocytoma

| MW (kDa)   |      |       |    |      |     |        |
| 97         | + + + | + + + | + + | + + + | + + | +      |
| 80         | + + + | + + + | + + | + + + | + + | +      |
| 64         | + + + | + + + | + + | + + + | + + | +      |
| 46         | + + + | + + + | + + | + + + | + + | +      |
| 31         | + + + | + + + | + + | + + + | + + | +      |
| 22         | + + + | + + + | + + | + + + | + + | +      |
| 15         | + + + | + + + | + + | + + + | + + | +      |

+++, Strong signal; ++, moderate signal; +, weak signal; –, no signal; MW, molecular weight.
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 were less expressed 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.

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.
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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