Granins and their derived peptides in normal and tumoral chromaffin tissue: Implications for the diagnosis and prognosis of pheochromocytoma

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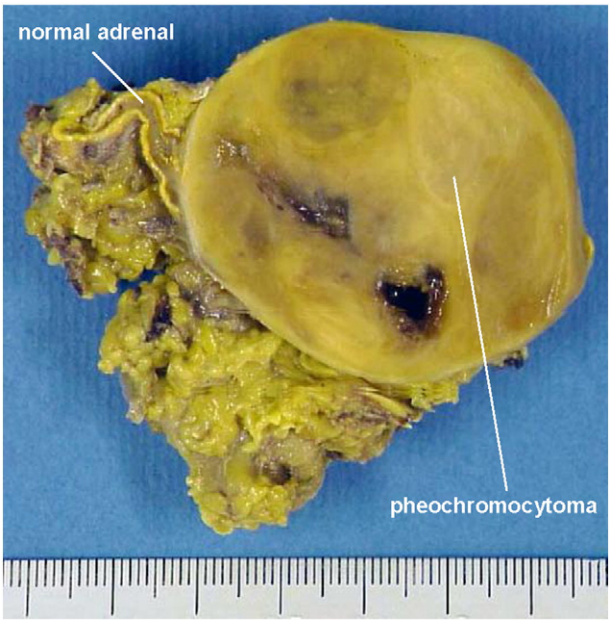
1. Introduction

The granin proteins are widely distributed in secretory granules of endocrine, neuroendocrine and nerve cells [1–3], and have also been described in other secreting cells like exocrine cells, cardiomyocytes or immune system cells [4–6]. At present, the granin family can loosely be divided into two groups, the chromogranins (Cg) and the secretogranins (Sg), distinguishable by the presence of a disulfide-bonded loop at the N-terminus of Cg but not in Sg proteins [7,8]. They constitute a family of acidic glycoproteins whose major members are chromogranin A (CgA), which was first isolated from chromaffin cells of the adrenal medulla [9]; chromogranin B (CgB, previously also called secretogranin I), initially characterized in a rat pheochromocytoma cell line [10]; and secretogranin II (SgII, previously also called chromogranin C), which was originally described in the anterior pituitary [11]. The granin family has further expanded to include other members such as secretogranin III (SgIII or 1B1075) [12], secretogranin IV (SgIV or HISL-19) [13], secretogranin V (SgV or 7B2) [14], secretogranin VI (SgVI or NESP55) [15], secretogranin VII (SgVII or VGF) [8,16] and proSAAS [17].

Granins are characterized by a high proportion of acidic amino acids (aspartic and glutamic acids) that confers the acidic nature of these proteins. This property enables them to play a role in the formation of secretory vesicles and sequestration of hormones in neuroendocrine cells [18–20]. In addition, granins encompass in their sequence numerous pairs of basic amino acids which are potential cleavage sites for prohormone convertases and which may give rise to bioactive peptides through post-translational proteolytic processing. Indeed, numerous studies have shown that peptides derived from granin maturation occur in various tissues and exhibit autocrine, paracrine, or endocrine activities [21,22]. The ubiquitous distribution of granins in endocrine and neuroendocrine tissues from which they are secreted into the bloodstream, makes granins useful markers of normal and tumoral neuroendocrine cells. In fact, multiple studies have documented the clinical value of detecting granins and their derived peptides in tissues and measuring their circulating levels [7]. In particular, measurement of CgA levels in plasma can be used to diagnose or monitor the progression of neuroendocrine tumors [23]. The highest accuracy has been observed in tumors characterized by an intense secretory activity, although the specificity and sensitivity remain high also in non-functioning tumors [24].

Among neuroendocrine tumors, pheochromocytomas are rare catecholamine-producing neoplasms primarily (80–85%) arising from chromaffin cells of the adrenal medulla or extra-adrenal paraganglia (Fig. 1). Tumors from extra-adrenal location are referred to as paragangliomas. Most of pheochromocytomas occur sporadically but approximately 25% result from germline mutations in 6 genes identified to date. Mutations of the VHL tumor suppressor gene predispose to the development of von Hippel–Lindau (VHL) syndrome, those of NF1 gene to von Recklinghausen's syndrome, and those of the RET proto-oncogene to multiple endocrine neoplasia (MEN) type 2A and 2B [25]. More recently, mutations identified in genes encoding succinate dehydrogenase (SDH) subunits B, C and D have been shown to predispose to familial paraganglioma syndromes [26]. Clinical presentation of pheochromocytoma can vary greatly, with similar signs and symptoms produced by many other clinical conditions. Most but not all the clinical manifestations of pheochromocytoma are due to the indirect actions of secreted catecholamines. Hypertension, tachycardia, pallor, headache and feeling of panic or anxiety, usually dominate the clinical presentation. Metabolic effects include hyperglycemia, lactic acidosis and weight loss. Less common signs and symptoms are nausea, fever and flushing [27]. Despite the increasing availability of molecular diagnostic and prognostic markers, it remains impossible to predict the development of malignant disease, based on histological findings in a resected tumor. Only the presence of metastases of tumoral chromaffin tissue establishes a definite diagnosis of malignant pheochromocytoma [27,28]. It should be noted that the incidence of pheochromocytomas is only 2–8 cases per 1,000,000 subjects and that malignant pheochromocytomas only represent about 10% of all pheochromocytomas.

In this review, we have explored the literature concerning the occurrence of granins in normal and tumoral chromaffin cells, and the



clinical usefulness of these proteins and their derived peptides for the diagnosis and the prognosis of pheochromocytomas.

2. Chromogranin A and derived peptides in pheochromocytoma

2.1. Chromogranin A

CgA is the major soluble protein, co-stored and co-released with catecholamines from storage vesicles of the adrenal medulla (Fig. 2). In 1983, using a radioimmunoassay (RIA) O'Connor and colleagues described the widespread distribution of CgA in polypeptide hormone-producing tissues and particularly in the medulla of the adrenal gland [29]. Thereafter, the ubiquitous distribution of CgA in bovine tissues was investigated by immunohistochemistry, showing the occurrence of a specific CgA immunostaining in adrenal medullary chromaffin cells [30]. Immunohistochemical labeling for CgA was then demonstrated in normal human adrenomedullary chromaffin cells, as well as in pheochromocytoma cells [31] (Table 1). O'Connor and Bernstein [32] were the first to report the measurement of immunoreactive CgA in human plasma. They have shown that in patients with pheochromocytoma, CgA concentration was higher than in healthy volunteers (1614 ± 408 ng/ml vs 129 ± 12 ng/ml, respectively). Accumulated data, thereafter, have confirmed high concentrations of plasma or serum CgA in patients with pheochromocytoma, and numerous assays have been developed using different markers (enzymatic or luminescent), other standard peptide (bovine CgA) or other antibodies (polyclonal, monoclonal or mixed system) [33,34]. Interestingly, Cleary and collaborators [35] showed that pheochromocytoma tissues from MEN2 patients expressed substantially more CgA than tumors from VHL patients at both the mRNA and protein levels. Moreover, they indicated that, relative to the increase in plasma catecholamines, patients with pheochromocytoma associated with MEN2 had higher plasma concentrations of CgA than those with tumors in VHL syndrome.

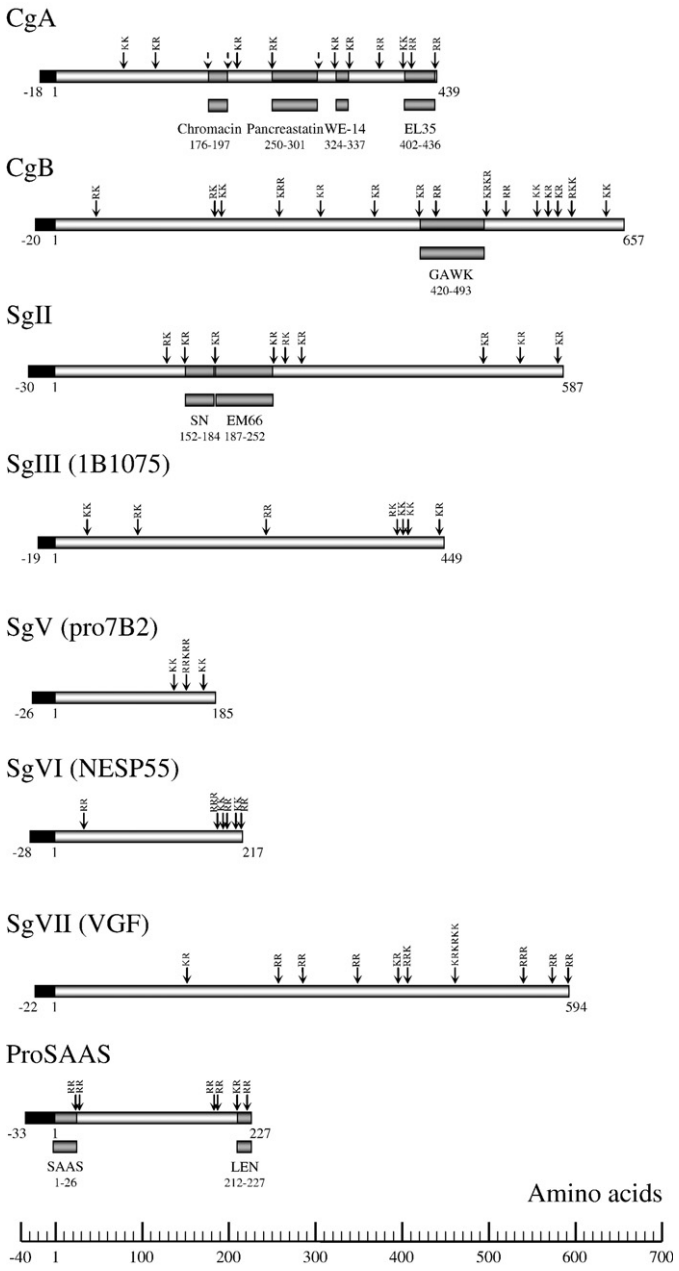


Fig. 2. Schematic representation of human granins and their derived peptides. Only granins and their processing products mentioned in the present review because of their presence in normal and/or tumoral chromaffin cells, or in patients with pheochromocytoma, are indicated. The *black boxes* correspond to signal peptides, *full arrows* indicate multibasic cleavage sites and *dotted arrows* indicate other cleavage sites. CgA, chromogranin A; CgB, chromogranin B; SgII–VII, secretogranin II–VII; K, lysine residue; R, arginine residue. The number of amino acids contained in the signal peptides, granins and derived peptides is indicated. No data are available for the sequence of the SgIV (HISL-19) protein.

CgA has been proposed as an alternative diagnostic test to catecholamines for the detection of pheochromocytoma, because its measurement in plasma is not substantially altered by drugs commonly used to diagnose or treat pheochromocytoma, such as phentolamine, tyramine, clonidine, and metoprolol [36]. Hsiao and collaborators [36,37] first examined the value of CgA determination for the diagnosis of pheochromocytoma and reported a diagnostic sensitivity of 83% and a specificity of 96% (Table 1, see the legend for the definition of the terms sensitivity and specificity). Similar results were obtained by Nobels et al. [38] while studying a relatively small number of cases ($n = 9$). It was found that the accuracy of plasma CgA measurement for the diagnosis of pheochromocytoma was lower than that of catecholamines or metanephrines [39]. These authors concluded that CgA determination did not offer additional information with respect to the other markers currently used in diagnostic setting of pheochromocytoma. However, Stridsberg and Husebye [40] reported that elevated CgA was observed in 90% of cases, and that no correlation was found between plasma CgA and urine adrenaline or noradrenaline, indicating that CgA could be considered as an independent biomarker for pheochromocytoma. They proposed that the combined assessment of blood CgA and urinary catecholamines could improve the diagnosis of pheochromocytoma. Similarly, Giovanella et al. [41] reported that measurement of circulating CgA displayed a higher sensitivity (100%), specificity (96%) and accuracy (96%) than all other tests performed (*i.e.* 24-hour urinary adrenaline, noradrenaline, vanillylmandelic acid and metanephrines), and concluded that the CgA assay might be used as a single test for the diagnosis of pheochromocytoma (Table 1). D'Herbomez et al. [42] reported a 90.8% concordance between CgA levels and ^{131}I-MIBG scintigraphy data and showed that a CgA level in the reference range is highly predictive of normal scintigraphy findings. More recently, the same authors [43] investigated in a group of 66 pheochromocytoma patients, the performance of several markers currently used for the diagnosis of this tumor. They showed that the more sensitive variables were plasma and urinary normetanephrines and plasma CgA. Finally, other authors have reported a high sensitivity of CgA assays and confirmed that combination with other tests like urinary catecholamines or metanephrines, increases the sensitivity for the diagnosis of chromaffin cell tumors [44,45] (Table 1).

Although CgA is a reliable sensitive marker for the diagnosis of pheochromocytoma, it has a poor diagnostic specificity [36,46–48]. This is in part due to the fact that the kidneys play a major role in the clearance of CgA from the circulation, so that even mild degrees of renal impairment can lead to a significant increase (false positive) in serum concentration of CgA. Canale and Bravo [49] indicated that in hypertensive patients with creatinine clearance less than 80 ml/min,

Table 1
Detection of granins and derived peptides in normal and tumoral chromaffin cells, and in circulation. Indication of the test assay sensitivity for the diagnosis of pheochromocytoma.

		Distribution				Test assay sensitivity**
		Normal chromaffin cells		Tumoral chromaffin cells (human pheochromocytoma)	Circulation	
CgA	CgA	+	h, p, b, m, r	+	+	71%–100%*
	WE-14	+	h, r	+	+	84%(n = 25)[66]
	EL35	+	h, r	+	ND	ND
	PST	+	h, pg	+	+	43%(n = 21)[40]
	chromacin	+	b	+	ND	ND
CgB	CgB	+	h, b, r	+	+	81%(n = 21)[40]
	GAWK	+	h, b	+	+	56%$GAWK_{1-17}$(n = 9)[77] 33%$GAWK_{20-38}$(n = 12)
SgII	SgII	+	h, p, b, d, m, r	+	+	ND
	SN	+	h, b, r	+	+	78%(n = 9)[87]
	EM66	+	h, b, r	+	+	92%(n = 12)[66]
SgIII (1B1075)		−		−	+	ND
SgIV (HISL-19)		+	h	ND	+	ND
SgV (7B2)		+	h, b, r	+	+	ND
SgVI (NESP55)		+	h,b, r	+	ND	ND
SgVII (VGF)		+	h, m, r	+	ND	ND
ProSAAS	proSAAS	+	h, m, r	PC12 cells	ND	ND
	SAAS	+	r	ND	ND	ND
	LEN	+	r	ND	ND	ND

A *plus* sign indicates the presence of the protein, granin-derived peptide and/or messenger RNA; a *minus* sign indicates absence or undetectable levels. b, bovine; CgA, chromogranin A; CgB, chromogranin B; d, dog; h, human; m, mouse; ND, not determined; p, primate; pg, pig; PST, pancreastatin; r, rat; SgII–VII, secretogranin II–VII; SN, secretoneurin. *The CgA sensitivity test assay for the diagnosis of pheochromocytoma differs according to the following studies: 71% (n = 45) [39]; 83% (n = 29) [36]; 86% (n = 50) [49]; 89% (n = 9) [38]; 90% (n = 21) [40]; 91% (n = 22) [45]; 95% (n = 44) [44]; 100% (n = 15) [41]. **_Sensitivity_: refers to the probability for a (diagnostic) test to be positive in a population of individuals with the target disorder (tumor, disease…) = true-positive / true-positive + false-negative. *Specificity*: refers to the probability for a (diagnostic) test to be negative in a population of individuals without the target disorder = true-negative / true-negative + false-positive. *False-negative*: individuals with the target disorder and negative for the test; *False-positive*: individuals without the target disorder and positive for the test; *True-negative*: individuals without the target disorder and negative for the test; *True-positive*: individuals with the target disorder and positive for the test.

the specificity of the CgA test assay for the diagnosis of pheochromocytoma was only 50% with a positive predictive value of 38%. However, the authors reported that combining CgA with plasma catecholamines provided a lower sensitivity, but excellent specificity, accuracy, and positive predictive value. In clinical practice, there is another major drawback of CgA measurement, *i.e.* elevated levels of CgA are common in patients under treatment with proton pump inhibitors, although no neuroendocrine tumor is present [48,50–52]. It was shown that patients taking proton pump inhibitors alone or in combination with antihypertensive medications had significantly higher CgA levels (mean 635 ng/ml) than subjects without any treatment (mean 174 ng/ml; $P<0.001$) [48]. Other reasons for non-tumor related increase of CgA are type A gastritis and heart failure [53,54]. Currently, CgA is the only granin whose assay is used routinely by clinicians for the diagnosis of neuroendocrine tumors. However, Stridsberg and collaborators [55] compared the usefulness of three commercial kits (CGA-RIA CT; CIS bio international, Gif-sur-Yvette cedex, France; DAKO Chromogranin A ELISA kit; DAKO A/S, Glostrup, Denmark; and CgA EuroDiagnostica, Malmö, Sweden) in different types of neuroendocrine tumors including pheochromocytomas and showed that, depending on the kit used, the sensitivity of the CgA test varied between 67 and 93%. Thus, in addition to false positive, plasma measurement of CgA may also produce false-negative test results in the diagnosis of neuroendocrine tumors, whose rate may depend on the CgA test used.

Several authors [36,37,41,42,44,56] observed a link between circulating CgA concentration and tumor burden, suggesting that CgA could be a good predictor of tumor size and catecholamine secretion. In contrast, low-secreting or non-functioning chromaffin tumors such as head and neck paragangliomas may go undetected when assessed for CgA. The granin can also be used in the follow-up of patients after resection of tumor and as a prognostic marker. Indeed, it was shown that CgA levels fall to normal shortly after removal of the tumor [24,36,37,42,45,56–58]. Rao and co-workers [59] observed that CgA levels were significantly different in benign vs malignant pheochromocytomas. Indeed, plasma CgA rose progressively from control subjects (48.0 ± 3.0 ng/ml) to patients with benign (188 ± 40.5 ng/ml) or malignant tumor (2932 ± 960 ng/ml). However, these results were not confirmed in another study which reported similar CgA levels in patients with benign or malignant neoplasms [45]. Nevertheless, Bilek et al. [56] found a significant relationship between plasma CgA concentrations and the PASS score [60] rating the malignancy of pheochromocytoma. Moreover, while performed on a low number of tumors, an immunohistochemical study using six CgA region-specific antibodies reported that the peptide expression pattern differed between benign and malignant pheochromocytomas. The benign neoplastic cells exhibited immunoreactivity to all region-specific antibodies, whereas those of the malignant pheochromocytomas expressed immunoreactivity to only one fragment ($CgA_{176-195}$) [61]. Finally, CgA may also be indicative of response to therapy in patients with malignant pheochromocytoma. It was shown that, in 5 responders to chemotherapy, there was a significant decline in CgA levels, whereas in 4 nonresponders CgA concentrations did not change, CgA varying longitudinally with tumor response and relapse [59].

2.2. WE-14

WE-14 is a 14 amino-acid long peptide derived from the proteolytic processing of CgA (human $CgA_{324-337}$) and was first isolated from ileal carcinoid tumor and pheochromocytoma tissues [62,63] (Fig. 2). WE-14 is widely distributed in neuroendocrine tissues including rat and human chromaffin cells [64]. Moreover, WE-14 immunostaining was also observed in tumoral chromaffin cells [65]. HPLC analysis combined with RIA detection demonstrated that WE-14 was present as a single immunoreactive peak in pheochromocytoma extracts. Measurement of circulating levels of WE-14 revealed significantly higher concentrations in patients with pheochromocytoma compared to healthy volunteers, the median preoperative level of WE-14 being about 4-fold higher [66]. *In vitro*, using cultured cells from a benign pheochromocytoma, it was demonstrated that metoclopramide, a gastroprokinetic and antiemetic agent, induced a concentration-dependent increase in WE-14 production with an EC_{50} reaching 3.84×10^{-8} M, and E_{max} of 78 ± 12% [67]. Moreover, the WE-14 response to metoclopramide was inhibited by

GR 113808 (10^{-7} M), a 5-HT4 receptor antagonist. Cisapride, a 5-HT4 receptor agonist, and dopamine were also shown to exert stimulatory effects on WE-14 release from pheochromocytoma cells [67]. These observations indicate that the levels of CgA-derived peptides may vary in pheochromocytomas in response to therapeutic interventions.

2.3. EL35

EL35 (human $CgA_{402–436}$) is a well-conserved peptide derived from the C-terminal domain of CgA (Fig. 2). Consistent with WE-14, EL35-like labeling is found in chromaffin cells of the rat and human normal adrenal glands as well as in benign pheochromocytomas [65]. However, HPLC characterization revealed only minute amounts of authentic EL35 in pheochromocytoma extracts while other EL35-immunoreactive forms were observed. The identity of this immunoreactive material was not determined but it should be mentioned that EL35 possesses an internal pair of basic residues which could be cleaved to give rise to shorter peptides. Therefore, the proteolytic processing of CgA to generate this peptide could be altered during tumorigenesis [65].

2.4. Pancreastatin

Pancreastatin (PST, human $CgA_{250–301}$) is a 52 amino-acid peptide located in the internal part of the CgA sequence, showing a low sequence similarity in vertebrate species [21] (Fig. 2). Using an antibody directed against synthetic porcine PST, Bishop et al. [68] reported the occurrence of PST-like immunoreactivity in a series of human neuroendocrine tumors. PST levels have been determined through RIA measurement in pheochromocytomas (from 8.0 to 69.0 pmol/g). However, no labeling could be observed by immunocytochemistry in the same study. This discrepancy was ascribed to the fact that the antiserum to PST was raised against the porcine peptide. The development of a specific assay for the measurement of circulating human PST allowed Stridsberg and Husebye [40] to investigate the usefulness of this peptide for the diagnosis of patients with pheochromocytoma. They found a low diagnostic sensitivity for this tumor (43%), since plasma PST levels were elevated in only nine out of twenty one patients (Table 1). This finding does not speak for the usefulness of PST as a marker for the diagnosis of pheochromocytoma.

2.5. Chromacin

Using antibodies directed against six regions of the CgA molecule including the chromacin peptide (human $CgA_{176–197}$) (Fig. 2), Portela-Gomes et al. [61] described a positive labeling for this CgA region in different neuroendocrine tumors studied including pheochromocytomas. Moreover, semi-quantitative analysis revealed a more intense chromacin-specific labeling in benign than in malignant pheochromocytomas, but this finding was not confirmed by other biochemical analyses.

3. Chromogranin B and derived peptides in pheochromocytoma

3.1. Chromogranin B

CgB is an abundant secretory protein found in a large variety of endocrine cells and neurons [69] (Fig. 2). CgB has been described in chromaffin cells of different mammalian species [70,71] and in pheochromocytoma extracts [72]. In benign pheochromocytomas, CgB was consistently detected, whereas in two out of three malignant tumors, the granin protein was absent [73]. In pheochromocytomas, while variations in the expression pattern occurred, no significant semi-quantitative differences were noted between benign and malignant tumors [74]. Nevertheless, in all malignant pheochromocytomas, the antibodies raised against the C-terminal region of CgB revealed a noticeable population of immunoreactive large spindle-shaped tumor cells, characterized by elongated processes. Interestingly, this cell type was only observed in one out of 25 benign tumors. Therefore, the authors suggested that the use of antibodies raised against epitopes of the C-terminal region of CgB could facilitate the diagnosis of malignant pheochromocytomas [74]. Stridsberg and Husebye [40] developed a specific assay for the measurement of circulating CgB, in order to investigate the usefulness of this protein for the diagnosis of patients with pheochromocytoma. They found a diagnostic sensitivity of the CgB test assay of 81%, the granin level being elevated in 17 out of 21 patients with pheochromocytoma (Table 1). While the CgA levels fell to normal shortly after the tumor was removed, the CgB levels decreased towards normal over the course of several days [40].

3.2. GAWK

GAWK (human $CgB_{420–493}$) is a 74 amino-acid peptide originally isolated from human pituitary gland extracts [75,76] (Fig. 2). Bishop et al. [77] have shown that GAWK-like immunoreactivity is present in chromaffin cells of the human adrenal medulla. Using two different antibodies (directed against the $GAWK_{1–17}$ and $GAWK_{20–38}$ fragments), a consistent positive immunoreactive staining was seen in all pheochromocytomas studied ($n = 6$) [78]. In contrast, only antibodies against $GAWK_{20–38}$ yielded an immunostaining in cells of normal adrenal medulla. RIA quantification revealed that $GAWK_{20–38}$ and $GAWK_{1–17}$ concentrations were about 15-fold higher in tumoral compared to normal chromaffin cells [78]. The GAWK levels were at least 10 times higher in pheochromocytomas than in any other GAWK-producing tumor. While circulating $GAWK_{1–17}$ levels were systematically more elevated in pheochromocytoma patients than in healthy volunteers (121 ± 66 pmol/l vs 7.0 ± 1.1 pmol/l), this was not the case for the $GAWK_{20–38}$ peptide (114 ± 19 pmol/l vs 74 ± 5.0 pmol/l) [78] (Table 1).

4. Secretogranin II and derived peptides in pheochromocytoma

4.1. Secretogranin II

SgII was observed in human adrenomedullary tissue and pheochromocytomas [71,73,79]. In the tumors, while variations in the staining pattern with different SgII antibodies were observed, no significant semi-quantitative differences were noted between benign and malignant neoplasms [74]. However, these authors described SgII-immunoreactive large spindle-shaped pheochromocytoma cells preferentially labeled in malignant tumors, as previously observed with CgB labeling. Guillemot et al. [80] showed that SgII mRNA was overexpressed in pheochromocytoma compared to non-tumoral chromaffin tissue and in benign compared to malignant tumors. In addition, western blot quantification revealed that the SgII protein was significantly more abundant in benign than in malignant neoplasms. Using region-specific antisera, Stridsberg et al. [81] showed differential occurrence of SgII-derived fragments in the plasma of pheochromocytoma patients.

4.2. Secretoneurin

The SgII-derived peptide SN (human $SgII_{152–184}$) is a highly conserved 33 amino-acid peptide [82,83] (Fig. 2) that was initially described in the rat and human adrenal medulla [84,85]. It was shown that SgII processing to yield SN was limited in the adrenal medulla and pituitary compared to other organs. Ischia et al. [86] determined the levels of SN in the serum of healthy subjects and patients suffering of pheochromocytoma. They first showed that the mean serum concentration of SN in 42 controls was 22.2 ± 1.1 fmol/ml (range 9.1–37.9). They observed that SN levels were age-dependent with higher concentrations in newborns followed by a continuous decline until 12 years, where adult levels were already reached. It was shown

that the contribution of the adrenal gland to serum SN-immunoreactivity was limited since, in patients with unilateral or bilateral adrenalectomy, no significant reduction in serum SN was observed [86]. In patients with pheochromocytoma, serum SN levels were elevated up to 5-fold (99.9 ± 25.6 fmol/ml) with only few patients within the normal range (Table 1). After surgical resection of the tumor, SN values returned to normal (18.3 ± 2.9 fmol/ml) [86]. Similar results were obtained by Stridsberg et al. [81], although these authors found concentrations of SN 10-fold higher in healthy volunteers with a reference range of 220 fmol/ml and concentrations up to 5400 fmol/ml in patients with pheochromocytoma. These differences could probably be ascribed to the different biological tools and methods used. However, these studies agree on the significant elevation of SN in pheochromocytoma patients.

4.3. EM66

EM66 (human $SgII_{187-252}$) is a 66 amino-acid long peptide that was initially identify by us (Fig. 2) [87,88]. We demonstrated that EM66 was produced in chromaffin cells of the adult and fetal human adrenal gland [89]. Subsequently, the presence of EM66-immunoreactivity was shown in rat and bovine adrenochromaffin cells [89,90] (Table 1). Immunohistochemical analysis demonstrated the occurrence of authentic EM66 in both benign and malignant pheochromocytomas [91]. RIA quantification revealed that a low EM66 concentration in pheochromocytoma was associated with a malignant status of this tumor [80,91]. It was also shown that pheochromocytes in primary culture had the ability to release high basal levels of EM66, suggesting that, *in vivo*, the peptide is secreted from the tumor in the general circulation [92]. In accordance, RIA determination revealed that plasma levels of EM66 were significantly higher in patients with pheochromocytoma compared to healthy volunteers (median 2.6, [1.9–3.7] ng/ml vs 26.9 [7.3–44] ng/ml, respectively) and returned to normal values after removal of the tumor [92]. In contrast, urinary EM66 levels in patients with pheochromocytoma were not significantly different from those of controls. Stridsberg et al. [81] found no change of EM66-like concentrations in patients with pheochromocytoma (see Section 4.1). This discrepancy may be ascribed to the antiserum used recognizing $SgII_{225-242}$ *i.e.* C-terminal part of EM66, and/or to the low number of cases studied (n = 3). As observed for the WE-14 peptide (see Section 2.2), metoclopramide, cisapride and dopamine dose-dependently stimulated EM66 release from cultured pheochromocytoma cells [67], again indicating that the levels of granin peptides are prone to variation under therapeutic treatment, at least with drugs targeting aminergic systems.

5. Other granins and their derived peptides in pheochromocytoma

While less studied, secretogranins III–VII and proSAAS also belong to the granin family and are characterized by numerous pairs of basic amino acids which may serve as potential cleavage sites for processing by the co-stored prohormone converting enzymes PC1/3 and PC2 in secretory granules [8] (Fig. 2). However, their presence and their use as circulating markers for pheochromocytomas have not been extensively explored (Table 1).

5.1. SgIII (or 1B1075)

SgIII is distinct from other granins as it is not found either in the adrenal gland or in pheochromocytomas [81].

5.2. SgIV (or HISL-19)

SgIV is a protein expressed by neuronal and peptide hormone-producing cells which shares many biochemical and molecular key features with the granin family [13]. Few studies reported the presence of SgIV in human adrenal medulla and in pheochromocytoma [13,93,94]. Immunoelectron microscopic analysis revealed SgIV immunoreactivity in secretory granules, which was apparently more pronounced in malignant compared with benign pheochromocytomas [94]. No data are available concerning circulating SgIV in pheochromocytoma patients (Table 1).

5.3. SgV (or 7B2)

7B2 is an acidic protein contained in most neuroendocrine cells. The presence of 7B2 was demonstrated in chromaffin tissue of the normal adrenal gland [95–98] and also in pheochromocytoma [98,99] (Table 1). In human, Natori and co-workers [100] determined plasma 7B2 levels in healthy volunteers and in patients with pheochromocytoma. They measured significantly higher plasma 7B2 concentrations in patients with pheochromocytoma than those in age-matched normal subjects (221 ± 82.8 ng/l vs 55.8 ± 1.2 ng/l).

5.4. SgVI (or NESP55)

NESP55 (neuroendocrine secretory protein of MW 55,000) is a soluble, acidic, heat-stable secretory protein [101] (Fig. 2) which was described in the rat and bovine adrenal medulla with a preferential localization in adrenaline-synthesizing cells [15,102]. NESP55 immunoreactivity was also shown in pheochromocytomas, with the same strong labeling pattern in both benign and malignant tumors [103–105] (Table 1).

5.5. SgVII (or VGF) and proSAAS

SgVII/VGF and proSAAS represent the most recently characterized members of the granin family (Fig. 2). VGF was originally identified as a neurotrophin-regulated gene product in PC12 cells [16]. ProSAAS was identified as the precursor of several peptides that accumulate in the obese, carboxypeptidase E-deficient Cpefat/fat mouse, and was shown to inhibit prohormone convertase 1 activity [17]. VGF and proSAAS are stored in and released from secretory vesicles and are expressed widely in nervous, endocrine, and neuroendocrine tissues [17,106,107]. Variable proVGF-immunoreactive fragments were observed in human adult adrenal medulla and in pheochromocytomas [108]. ProSAAS mRNA is primarily expressed in the brain but also occurs in endocrine organs and cells including the adrenal gland and the rat pheochromocytoma PC12 cell line [17] (Table 1). Two proSAAS-derived peptides (SAAS and LEN) were detected in the rat adrenal medulla [109] (Fig. 2).

6. Concluding remarks

Malignant pheochromocytomas are orphan diseases for which there exist a difficulty for the management and a disparity in clinical practices. To date, the universally admitted criterion of malignancy for a pheochromocytoma is the occurrence of metastasis [110,111]. Therefore, numerous efforts have been undertaken to discover reliable tools for the early diagnosis and prognosis of malignant pheochromocytomas. While pathological analysis utilizing multiple histological criteria appears to hold some value for distinguishing benign from malignant disease, these evaluations are not practiced routinely as predictors of malignancy [60,112,113]. Among pheochromocytoma susceptibility genes, it is now established that SDHB mutation is associated with the highest risk of malignancy [114]. It has also been shown that malignant pheochromocytomas are characterized by an enhanced production of noradrenaline and that extra-adrenal (paragangliomas) tumors secrete dopamine [115]. A global and promising approach of gene expression profiling of benign and malignant pheochromocytomas, has allowed to identify a set of genes that could discriminate the two types of tumors [114,116]. As indicated in the present review, except SgIII, all members

of the granin family and several of their derived peptides have been shown to be present in normal chromaffin cell and in pheochromocytomas (Table 1). However, to date, CgA is the only granin currently used for the diagnosis of chromaffin cell tumors; a positive CgA immunostaining indicating the neuroendocrine nature of the neoplasm in general, without specificity for pheochromocytoma. Currently, besides CgA, other biochemical tests used for the diagnosis of pheochromocytoma are the measurement of plasma and urinary catecholamine metabolites (metanephrine and normetanephrine). While they offer a high sensitivity, these tests also have limitations. To date, a biochemical test alone is obviously not sufficient for the screening of pheochromocytoma. False-negative and false-positive results using biological tests remain a problem, leading to cost-effective and time-consuming additional imaging examinations [117,118]. It is therefore tempting to propose that the measurement of CgA and metanephrine levels should be combined with the determination of other granins or granin-derived peptides for the diagnosis/prognosis of pheochromocytoma.

CgA levels are correlated with tumor mass since malignant pheochromocytomas which are often more bulky than benign ones display higher CgA concentrations [43]. Thus, CgA may serve as a prognostic marker in certain conditions but there are several restrictions such as the existence of malignant non-secreting chromaffin tumors of the head and neck. Other drawbacks of CgA limit the clinical use of the protein (false-negative or false-positive test results) and therefore, there is a need to identify other granin or granin-derived peptides for the diagnosis and prognosis of pheochromocytomas. In this respect, CgB did not seem to have a better diagnostic sensitivity than CgA but was shown to be a complementary tool for the management of neuroendocrine tumors, since its concentrations were not elevated in patients with renal failure or under treatment with proton pump inhibitors [57]. It was also postulated by the group of Stridsberg and Portela-Gomes that the use of antibodies raised against epitopes of the C-terminal region of CgB could facilitate the diagnosis of malignant pheochromocytoma [74]. Our data showed that SgII mRNAs were down-regulated in malignant compared to benign pheochromocytomas and that malignancy was associated with reduced levels of SgII and SgII-derived peptides, in accordance with the low concentrations of EM66 found in malignant tumors [80,91]. Taken together, these observations strengthen the notion that granins other than CgA and their derived peptides may provide new diagnostic and prognostic markers of pheochromocytoma.

An important issue raised by A. Tischler, is to define more precisely the term "malignant" concerning pheochromocytoma/paraganglioma since the potential lethality of extensive local invasion is ignored by a definition of malignancy based only on metastasis [119]. He suggested that pheochromocytoma and extra-adrenal paraganglioma could be classified as "non-invasive", "minimally invasive" and "extensively invasive", in order to stratify the risk of metastasis and aggressiveness for a better management of patient treatment [119]. In this regard, the development of additional markers derived from granins and their peptides used solely or in combination, *i.e.* through array of different peptide-recognizing antibodies, may provide a mean to accurately discriminate different pheochromocytoma subtypes. However, a precise description of the relative affinity of each antibody raised against a granin-derived peptide towards the peptide itself and its precursor will be needed because this important information is not always available. Other challenges to the diagnosis and prognosis of pheochromocytoma will be to detect tumors that do not contain or produce excess catecholamines, metanephrines or CgA. Concerning the prognostic value of histopathology of pheochromocytoma tumors, to date there is no consensus on the reliability of the PASS score [60]. Further studies should combine histological (PASS), immunohistochemical (Ki67) and biochemical (adrenergic/noradrenergic, granins) features to predict metastatic potential of tumors [113,120,121]. However, it should be kept in mind that these different markers would carry different weights depending on the adrenal or extra-adrenal localization and the genetic background of the tumor [119]. In addition, chromaffin tumors are rare, heterogeneous, and further work aimed at identifying new predictive factors of malignancy should be performed with large and well characterized cohorts of patients. Finally, *in vitro* experiments, performed on tumor cell lines, suggest that CgA may affect tissue remodeling and tumor growth [122]. Therefore, in addition to their potential use as predictors for the diagnosis and prognosis, granin-derived peptides could also be considered for use in therapeutic strategies for the treatment of pheochromocytoma and other neuroendocrine tumors.

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