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Chromogranin-A as a serum marker for neuroendocrine tumors: comparison with neuron-specific enolase and correlation with immunohistochemical findings

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ABSTRACT: Background: *Chromogranin-A (Cg-A) is a 439-amino-acid protein contained in secretory granules of neuroendocrine cells, in addition to specific hormone peptides or neuropeptides. Since Cg-A is co-released with peptide hormones its serum concentration can be used as a marker of neuroendocrine tumors.*
Aim: *Evaluation of the analytical performance of a new IRMA method for Cg-A assay and of the clinical value of serum Cg-A and neuron-specific enolase (NSE) in neuroendocrine tumors. In addition, we compared the diagnostic usefulness of both Cg-A and NSE serum levels and their relationship to tissue expression.*
Patients and methods: *Initially we evaluated the analytical performance (intra- and interassay imprecision, dilution test and detection limit) of the Cg-A RIACT method (CIS Bio-International, Gif-sur-Yvette, France). We selected 50 patients affected by various histologically confirmed neuroendocrine tumors (NETs): ^{111}In-pentetreotide scan and helical computed tomography were employed to assess tumor extent. Cg-A and NSE were measured before surgery in serum samples of patients and 50 age-matched controls by IRMA methods. After surgery immunohistochemical stains for Cg-A and NSE were performed on surgical specimens of tumor tissue.*
Results: *Cg-A levels were significantly higher ($p<0.0001$) in patients with NETs than in healthy controls and we found a positive correlation between serum and tissue expression ($p<0.05$). Serum levels of Cg-A were also related to tumor extent ($p<0.05$) but in some cases we observed significant elevation of serum Cg-A in small, intensely immunoreactive NETs. ROC curve analysis showed better accuracy for serum Cg-A compared to NSE in the diagnosis of NETs, while no significant relationship was found between serum expression and immunostaining for NSE.*
Discussion: *Our results confirmed the biological and clinical significance of circulating Cg-A as an expression of granular content in neuroendocrine tissues and supported the complementary usefulness of serum Cg-A in the diagnosis and evaluation of NETs together with imaging modalities. (Int J Biol Markers, 1999; 14: 160-6)*

KEY WORDS: Chromogranin-A, Neuroendocrine tumors, Immunoradiometric assay

INTRODUCTION

Chromogranin A, a 439-amino-acid protein originally identified in chromaffin cells of the adrenal medulla, is contained in secretory granules of several neuroendocrine tissues. In fact, in addition to specific hormone peptides or neuropeptides, these granules also contain one or more chromogranin/secretogranin proteins (1). All of them belong to a unique family of secretory proteins which share many biochemical properties and the exclusive presence in neuroendocrine secretory granules. Three members of this family have been identified: chromogranin A (Cg-A), chromogranin B (Cg-B) or secretogranin I and chromogranin C (Cg-C) or secretogranin II (2,3). Cg-A shows the widest neuroendocrine expression and distribution and is present

also in some cells not expressing other granins (4). Recent findings suggest that Cg-A has different biological functions. It has been found to be the precursor of several peptides such as pancreastatin (5), vasostatin/betagranin (6) and chromostatin (7). In addition, Cg-A shows some intracellular functions: it modulates the proteolytic processing of hormones during their transport in neuroendocrine vesicles and it seems to be involved in mechanisms regulating peptide storage and secretion (8). Because of these biological and physiological characteristics, Cg-A has been widely used as an immunohistochemical marker of neuroendocrine neoplasms, and particularly of those tumors lacking the ability to produce hormones (9-11). Moreover, since Cg-A is co-released with peptide hormones, its serum concentration can be used as a marker of neuroendocrine tumor secretion (12), in addition to more traditional markers such as neuron-specific enolase (NSE), which, on the other hand, does not seem to be highly sensitive (13).

Using radioimmunological assay (RIA) and enzyme-linked immunosorbent assay (ELISA), some authors have demonstrated the clinical usefulness of Cg-A as a circulating marker of neuroendocrine neoplasms (14-16). Recently, a new immunoradiometric assay (IRMA) has been developed to detect circulating Cg-A. This study was designed to evaluate the analytical performance of a new IRMA method for Cg-A detection and, in addition, the diagnostic value of serum Cg-A in neuroendocrine tumors. We also compared the diagnostic usefulness of both Cg-A and NSE serum levels and their relationship to tissue expression.

MATERIALS AND METHODS

Serum samples were obtained from 50 subjects (24 males, 26 females; mean age 48 years (range 23-82 years)) with the following neuroendocrine tumors (NETs): 35 gastroenteropancreatic (GEP) neuroendocrine tumors (14 pancreatic, 5 gastric, 5 duodenal, 1 jejunal, 6 ileal, 3 appendiceal, 1 colorectal), 4 breast neuroendocrine carcinomas, 8 lung tumors (4 bronchial carcinoids, 4 small cell lung cancers), 3 medullary thyroid carcinomas. All diagnoses were histologically confirmed and serum was sampled before surgery. As control group we selected 50 age-matched healthy subjects.

Determination of tumor extent

Evaluation of tumor extent was performed using helical computed tomography images (CT) and [^{111}In-DTPA-D-Phe^{1}]-octreotide scan for somatostatin receptor scintigraphic visualization (SRS) (17). The tumor was considered limited when only a primary mass was detected and extensive when any other localization was demonstrated.

Immunoradiometric assay of Cg-A and NSE

Cg-A was measured in serum samples (stored at -80°C) by means of a newly developed immunoradiometric assay (IRMA) provided by CIS Bio-International (Gif-sur-Yvette, France). Cg-A RIACT is a solid-phase, two-site IRMA method: two monoclonal antibodies were prepared against steric remote sites on the Cg-A molecule. The first is coated on the solid phase (coated tube) and is used as catcher, while the second, radiolabeled with iodine-125, is used as tracer. The molecules or fragments of Cg-A present in the standards or the samples to be tested are "sandwiched" between the two antibodies. Following the formation of the coated catcher/Cg-A/tracer sandwich, the unbound tracer is removed by a washing step. The radioactivity bound to the tube is proportional to the concentration of Cg-A present in the sample.

NSE was detected by a commercially available IRMA method (NSE Prolifigen, AB Sangtec Medical, Bromma, Sweden) according to the manufacturer's instructions.

Evaluation of the analytical performance of the Cg-A assay

Intra and interassay imprecision

Imprecision was assessed using three different serum pools obtained from 10 healthy subjects (level A, normal), 10 patients affected by limited NET (level B, pathological 1) and 10 patients with diffuse NET (level C, pathological 2). Serum samples were tested either 10 times in the same series of assays (intra-assay imprecision) or in six different series (interassay imprecision). The results are summarized in Table I.

Dilution test

A sample with a known high concentration of Cg-A was sequentially diluted, with the recovery percentage ranging from 96% to 102% (R^2: 0.9993, $p<0.0001$) (Figure 1).

Detection limit

The detection limit is defined as the smallest detectable concentration different from zero and evaluated

TABLE I - INTRA- AND INTERASSAY IMPRECISION PROFILE OF Cg-A RIACT METHOD

	Intra-assay (n=10 replicates)			Interassay (n=6 series)		
	Mean	**SD**	**CV**	**Mean**	**SD**	**CV**
Level A	39.2	3.1	0.08	42.3	4.6	0.11
Level B	124.1	7.4	0.06	131.6	10.5	0.08
Level C	278.8	16.7	0.06	294.2	17.6	0.06

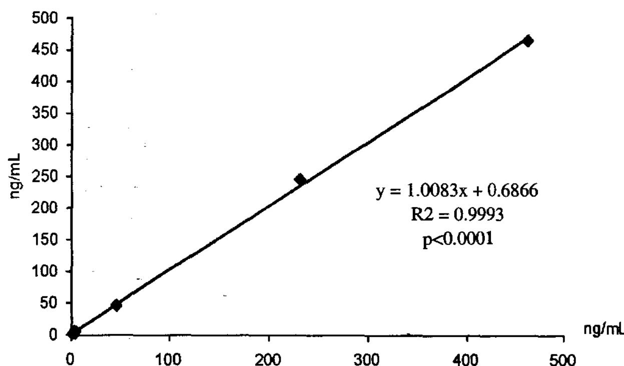

Fig. 1 - *Sequential dilution test on high concentration Cg-A serum.*

as standard zero plus three standard deviations. It has been determined as 2.1 ng/mL.

Immunohistochemistry

Immunohistochemical stains were performed on 35 formalin-fixed and paraffin-embedded tumor tissues, using the avidin-biotin complex (ABC) procedure according to a previously described method (18). Briefly, after inhibition of endogenous peroxidase activity, deparaffinized sections were incubated for 24 hours at 4° C with specific monoclonal antibodies directed against Cg-A (clone PHE5, Enzo Diagnostics, USA) and NSE (clone MIG-N3, Monosan, The Netherlands) at a dilution of 1:50 and 1:100, respectively. Subsequently, the sections were incubated for 1 hour with a 1:200 dilution of biotinylated antimouse immunoglobulins, followed by incubation for another hour with the avidin-biotin complex. Lastly, the sections were developed in 0.03% 3,3'-diaminobenzidine tetrahydrochloride and counterstained with Harris' hematoxylin.

Statistical analysis

Since the tumor markers were not normally distributed, we expressed the results as median and interquartile range and a non-parametric analysis was used. The differences between two independen groups were determined by means of the Mann-Whitney U-test. ROC curves were defined using marke serum levels of both controls and patients, establishing a relationship between sensitivity and specificity Areas under the ROC curves were calculated using a previously described method, while the Youden tes was applied to the ROC analysis to obtain the bes cutoff value for each marker (19). Immunohistochemical expression of Cg-A was semi-quantitatively expressed as the percentage of positive cells and the correlation between circulating levels and immunohistochemical expression of markers was evaluated by linear regression analysis. p values < 0.05 were considered statistically significant.

RESULTS

Diagnostic performance of Cg-A and comparison wit serum NSE

The serum concentrations of Cg-A and NSE were determined in patients with neuroendocrine tumors and compared to the marker levels of the control group. The marker distribution in patients with neuroendocrine tumors and in controls is illustrated in Figure 2. Cg-A levels were significantly higher (p<0.0001) in patients with neuroendocrine tumors than in health controls, whereas no significant difference betwee the two groups was observed for NSE. ROC curv analysis allowed the global evaluation of the marker in terms of accuracy: the areas under the curve (AUC for Cg-A and NSE were 0.82 (0.03) and 0.54 (0.05) respectively, indicating a better accuracy for Cg-A than for NSE (Fig. 3). The selected cutoff values wer 98 ng/mL for Cg-A and 16 µg/L for NSE and the relative sensitivity, specificity and overall accuracy of bot markers were 68% and 18%, 92% and 95%, 81% an 56% for Cg-A and NSE, respectively. The diagnosti performance of Cg-A and NSE in neuroendocrine tumor subgroups is illustrated in Table II.

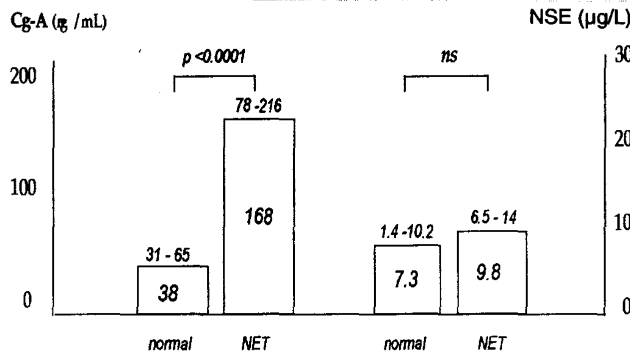

Fig. 2 - *Cg-A and NSE distribution in patients with neuroendocrine tumours (NET) and controls (ns= not significant).*

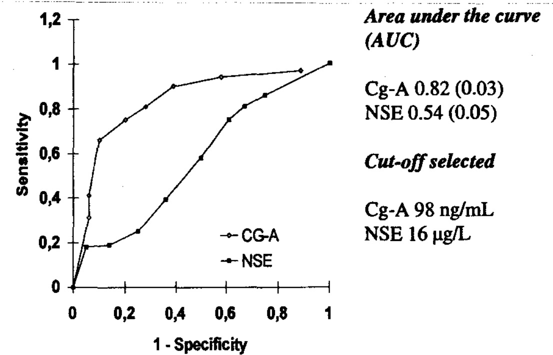

Fig. 3 - *ROC analysis and comparative evaluation of markers.*

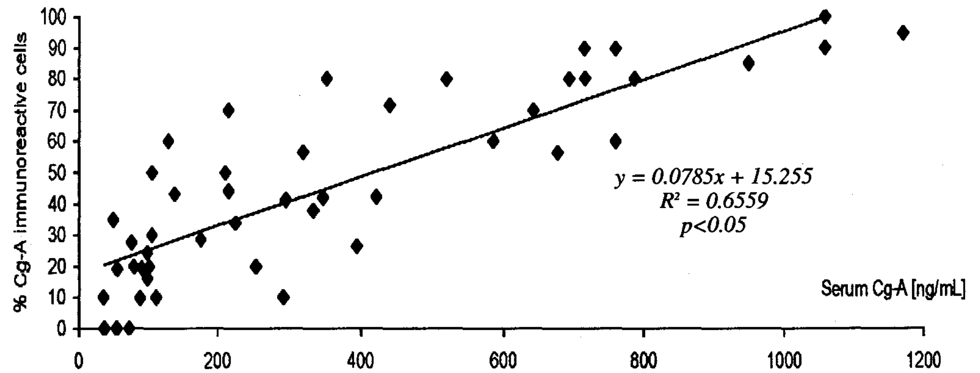

Fig. 4 - *Relationship between serum and tissue Cg-A expression.*

Relationship between circulating Cg-A and NSE and immunohistochemistry

We found a significant overall positive correlation between serum and tissue Cg-A (R^2 0.655, $p<0.05$), as shown in Figure 4, whereas no significant relationship was found between serum expression and immunostaining for NSE. All neuroendocrine tumors showed positive immunostaining for NSE, whereas only 9/50 patients with neuroendocrine tumors (4 SCLC, 2 bronchial carcinoids, 2 pancreatic NETs, 1 ileal NET) presented raised NSE serum levels.

Relationship of serum Cg-A and NSE with tumor extent

The overall distribution of Cg-A serum levels was significantly ($p<0.05$) different between limited (23 cases) and extensive (27 cases) neoplasms, being generally and more frequently higher in the latter (Table II). However, among the limited tumors we observed a Cg-A serum level highly above the cutoff in three cases, which were small in size (diameter less than 2 cm) but intensely Cg-A immunoreactive (Fig. 5). These neoplasms reached levels similar to those of some malignant metastatic tumors which, by contrast, were less immunoreactive to Cg-A. On the other hand, three cases of exensive disease showed normal concentrations of CgA in the serum (Table II).

No significant relationship between tumor extent and NSE serum levels was found.

DISCUSSION AND CONCLUSIONS

We evaluated a newly developed IRMA method to detect serum levels of Cg-A, based on two monoclonal antibodies directed against two contiguous epitopes within the median 145-245 human Cg-A sequence. This assay allows sensitive detection of total human Cg-A because dibasic cleavage sites present in the central domain of the molecule do not seem to be affected by degradation (20). The sampling procedures and the assay methodology are very simple and easy to perform. Our results confirmed that the Cg-A RIACT

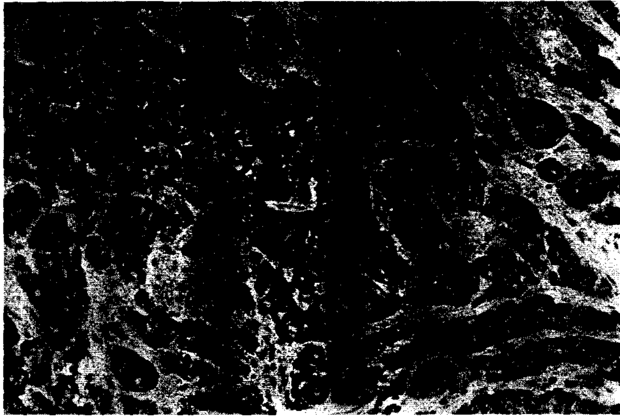

Fig. 5 - *Chromogranin-A immunoreactivity in an appendiceal well differentiated EC-cell tumour (avidin-biotin-peroxidase technique, X200).*

method shows good analytical performance. Serum Cg-A in the control group differed significantly from that of patients affected by neuroendocrine tumors and the overall diagnostic performance of Cg-A was clearly superior to that of NSE (21).

We obtained immunohistochemical stainings of neuroendocrine tumors and correlated serum Cg-A with its tissue expression.

Our results confirmed the biological and clinical significance of circulating Cg-A as an expression of granular content in neuroendocrine tumors and, consequently, as a serum marker of neuroendocrine differentiation. Moreover, as assessed in previous studies, our findings showed that serum levels of Cg-A were related to the extent of disease (9, 12). However, it is important to underline that the same high Cg-A serum values may result from either well-differentiated, densely granulated, localized neuroendocrine neoplasms or metastatic, poorly granulated neuroendocrine carcinomas. This finding suggests that, although Cg-A serum levels can be considered an useful marker of neuroendocrine tumors and may be employed in diagnosis, biological characterization and monitoring of the course of disease, in our opinion serum Cg-A alone should not be used in the staging of disease. In this setting the role of biomedical imaging and, particularly, octreoscan is still of essential importance.

In our study the serum levels of NSE, which were significantly elevated only in four small cell lung cancers, did not show any relationship with the presence of neuroendocrine tumors and with tumor extent, confirming the findings of other studies (12, 15). On the other hand, the neuroendocrine tumors showed positive immunostaining for NSE. This result may be explained considering the different cellular location of the two markers: in fact, while Cg-A is part of the neuroendocrine granule, NSE is a cytoplasmic enzyme. Generally, neuroendocrine tumors are relatively well differentiated and tumor cells partially retain their biological properties, including their secretory activity, so that Cg-A is actively secreted by the cells. By contrast, serum NSE elevation may be indicative of a lack of cell membrane integrity. With the exception of rare poorly differentiated neuroendocrine carcinomas, neuroendocrine

TABLE II - DIAGNOSTIC PERFORMANCE OF SERUM Cg-A AND NSE AMONG VARIOUS NEUROENDOCRINE TUMORS

Neuroendocrine tumors [illegible]	Limited disease (n=23)		Extensive disease (n=27)	
	Cg-A	NSE	Cg-A	NSE
Gastro-entero-pancreatic (n=35)				
Pancreas (n=14)	4/9	1/9	5/5	1/5
Stomach (n=5)	1/2	0/2	3/3	0/3
Duodenum (n=5)	1/2	0/2	3/3	0/3
Jejunum (n=1)	0	0	1/1	0/1
Ileum (n=6)	2/3	0/3	2/3	1/3
Appendix (n=3)	1/3	0/3	0	0
Colon-rectum (n=1)	0/1	0/1	0	0
Lung (n=8)				
Bronchial carcinoid (n=4)	0/2	1/2	1/2	1/2
Small cell lung cancer (n=4)	1/1	1/1	3/3	3/3
Breast (n=4)	0	0	3/4	0/4
Medullary thyroid cancer (n=3)	0	0	3/3	0/3
	10/23	3/23	24/27	6/27

tumors are generally well differentiated, lacking cell membrane lysis and tissue necrosis. According to this hypothesis serum NSE may be considered a marker of aggressive neoplasms and it should be preferentially employed in association with Cg-A serum detection in the biochemical evaluation of neuroendocrine tumors (22, 23).

In conclusion, we confirmed the complementary usefulness of serum detection of Cg-A in the diagnosis and evaluation of neuroendocrine tumors, together with imaging modalities and biochemical characterization of tumor secretion. Our findings also suggest that serum levels are directly related to Cg-A expression at the cellular level. Finally, we conclude that the RIACT method may be proposed as a simple and accurate method to quantitate circulating Cg-A concentrations.

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