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4. Pathological diagnosis and tumor markers

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ABSTRACT

Pulmonary and digestive neuroendocrine tumors (NETs) are a group of neoplasms whose incidence and prevalence has been constantly increasing over the last years thanks to the significant improvements in instrumental diagnostic techniques. Because NETs are extremely heterogeneous a correct histopathological diagnosis is essential for appropriate treatment. More specifically, the histopathological diagnosis of NETs can be regarded as a multistep: identification of the neuroendocrine nature of the neoplasm, determination of tumor grading; identification of unknown primary. Laboratory biomarkers for the study of gastroenteropancreatic neuroendocrine tumors include both specific markers and non-specific or general markers. At the moment, chromogranin A is the best available and most frequently used biomarker for the diagnosis of NETs, offering the highest overall sensitivity. CgA has also demonstrated some utility in the assessment of response to treatment and as indicator of tumor recurrence. Free full text available at www.tumorionline.it

1. Pathological diagnosis

1.1 Identification of the neuroendocrine nature of neoplasm

The neuroendocrine nature of neoplasm can be essentially deduced from the histological pattern, and from special staining techniques. The histological pattern of most endocrine tumors is characterised by a solid, trabecular or glandular arrangement of well-differentiated cells which may also form pseudorosettes or tubulo-acinar structures. In most cases, these morphological features are sufficiently distinctive of the endocrine nature of the tumor. At microscopic analysis, neuroendocrine neoplasms are characterized by monomorphism, uniformity of cell size, presence of polygonal cells and coarse cromatin. Although the determination of the endocrine nature is facilitated by the repeatability of this cytological appearance in the majority of endocrine tumors, the identification of the endocrine cell of origin is much more difficult because such morphological features do not vary according to the primary tumor site. A classification in cytological patterns has been developed, but it has a merely descriptive value and doesn't add any relevant information to prognosis or treatment. Much more useful from this point of view is the morphological distinction between the two major categories of well-differentiated and poorly-differentiated NETs. Well-differentiated NETs diplay a series of mixed solid-insular, pseudo-glandular and trabecular patterns, whereas poorly-differentiated NETs show a patternless arrangement with diffuse necrosis, marked cellular atypia and high mitotic rate (Figure 1A and 1B).

Once established, the histological diagnosis of NET must be confirmed by special staining techniques such as argentaffin staining by Masson and argyrophil staining by Grimelius, which is a general marker for neuroendocrine differentiation. However, these two methods have recently been mostly replaced by immunoistochemistry (ICH) using antibodies against general cytosolic and granular neuroendocrine markers. Among cytosolic markers, the most frequently used is the microvesicular marker synaptophysin, which is equally and diffusely expressed both in well-differentiated and poorly differentiated neoplasms. Instead isn't useful the staining for NSE and

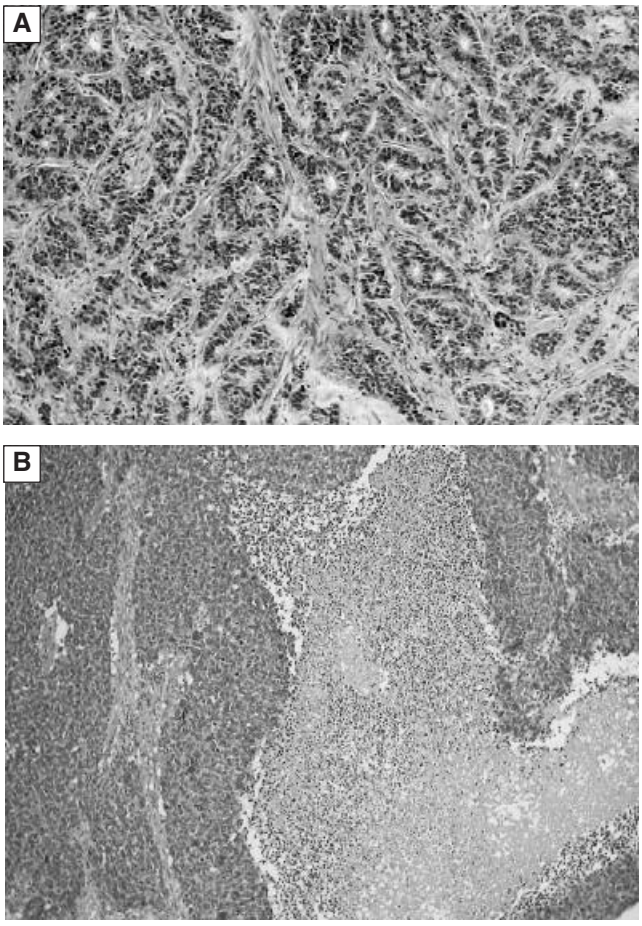

Figure 1 - Neuroendocrine nature growth pattern: well-differentiated endocrine carcinoma (A) and poorly differentiated endocrine carcinoma (B).

PGP9.5: those markers have shown many false positive results. The most frequently used granular markers are chromogranin A and B, which are highly expressed by well-differentiated neoplasms but are absent or only focally expressed in poorly-differentiated neoplasms. The immunoistochemical detection of general endocrine markers allows not only to confirm the endocrine nature of the tumor, irrespective of its grade of differentiation, but also to pose a differential diagnoses with other neoplasms, first of all GEP neoplasms of exocrine origin.

1.2 Determination of tumor differentiation-grading

Today the WHO classification still represents the backbone of NETs classification system and identifies two major categories: well-differentiated neuroendocrine tumors (WDETs) and well-differentiated neuroendocrine carcinomas (WDECs) on one hand, and poorly differentiated neuroendocrine carcinomas (PDECs) on the other. These large categories represent two different tumor entities and criteria used to identified well-differentiated lesions cannot be applied to the detection of poorly-differentiated ones. As previously mentioned, WDETs display a organoid (insular or trabecular) architecture, cellular monophormism, mild o absent cell atypia and a low mitotic index. This morphological pattern is present in all the WHO categories of well-differentiated tumors, i.e., tumors with benign behaviour, tumors with uncertain behaviour and carcinomas, and doesn't allow to further distinguish among well-differentiated subcatogories. More specific information about tumor behaviour can be obtained by the assessment of other morphological parameters, such as vascular invasion, tumor size, the invasion to muscolaris propria or beyond and the presence of distant metastases. Among morphological parameters, angioinvasion is the best predictor of survival and allows to further restrict diagnosis to one of the subcategories of WDETs.

1.3 Determination of tumor prognosis

The determination of tumor prognosis can be made by means of the proliferative index Ki-67 according to the ENETS grading system. Among neuroendocrine markers, Ki-67 has a role comparable to angioinvasion in the morphological parameters as an independent prognostic predictor for assessing the clinical outcome of individual patients. Ki-67 is a human nuclear cell-cycle associated protein whose expression is strictly associated with cell proliferation, being expressed in all active parts of the cell cycle (G1, S, G2, and mitosis) but not in resting cells. Today, Ki-67 detection by using the monoclonal antibody MIB-1 is widely use in routine pathology to measure the growth fraction of cells in human tumors. Specifically, Ki-67 value expresses the percentage of 2000 cells positive for MIB-1 in areas of highest nuclear labelling.

The prognostic value of vascular microinvasion and Ki-67 were clearly demonstrated by several studies. In a work by La Rosa *et al.*[1], which analysed a series of 61 non syndromic pancreatic endocrine tumours for macroscopic, histopathological and immunohistochemical variables potentially predictive of malignancy, vascular microinvasion and Ki-67 proliferative index were the most sensitive and specific prognostic variables, significantly affecting survival rate in 45 cases of well-differentiated tumors.

In another study by Rigaud *et al.*[2], it was demonstrated that Ki-67 has a predictivity comparable to that of an analisys of the allelotype on 16 pancreatic neuroendocrine tumors. In this study, the ploidy status was found to be significantly associated with outcome, with diploid tumors showing a significantly superior survival compared to diploid tumors. Specifically, all patients with aneuploid tumors and a Ki-67 index >2% died of the disease within 5 years, whereas all patients with euploid tumors and a Ki-67 index <2% were still alive after 10 years (P <0,0001). However, survival curves obtained for the ploidy status were comparable with those for Ki-67, demostrating the high prognostic value of this marker.

However, Ki-67 evalutation can vary according to tumor localization. For example, Ki-67 value is typically very low (constantly <1%) in intestinal neoplasms, where it has a limited role, while presents a greater variability in pancreas. A study by Pelosi *et al.*[3] on well-differentiated pancreatic tumors showed that survival changed considerably when Ki-67 cut-off was brought from the standard WHO value of 2% to 5%, with decreased percentage of cumulative survival in tumors showing Ki-67 index >5%. However, it's worth noting that there are some exceptions that do not follow all histological, immunoistochemical e grading parameters considered so far. A clear example is provided by well-differentiated rectal carcinoma. Rectal carcinoma diplays a morphological pattern typical of well-differentiated neoplasms, a positive immunostaining for synaptophysin and PP and a Ki-67 <1%. At the same time, it is negative for CrA because secretory granules located in this portion of the gastrointestinal tract do not produce this protein.

1.4 Identification of unknown primary

In a recent study, we analyzed by PCR microarray the gene expression profile of a uniform series of sporadic, non functioning (NF) pancreatic endocrine tumors (PETs) with progressive disease and their liver metastasis[4]. PETs were well distributed between well-differentiated and poorly differentiated. Thirteen NF PET samples (eight primaries and five liver metastases) from ten patients with progressive, metastatic disease, three cell lines (BON, QGP and CM) and four purified control islet samples were analyzed. Of the 990 individual dysregulated genes obtained comparing primary and metastatic lesions to islets, most had never been associated with PETs before. Considerably, when primary tumors were compared with metastatic lesions, no significant differentially expressed genes were found, suggesting an accumulation of most genetic abnormalities in the primary tumor. Given the striking similarity in the gene expression profiles of primary tumors and metastases, one would expect that mestastases maintain the phenotype of its originating organ. This observation provides the rationale basis for the detection of unknown primary by using immunoistochemical markers such as CDX-2, vesicular monoamine transporter 2 (Vmat-2) and serotonin for a suspected gastrointestinal (GI) origin, thyroid transcription factor-1 (TTF-1) for the lung, progesteron receptor (PR), insulin, glucagon and pancreatic polypeptide (PP) for the pancreas. Accordingly, a metastatic lesion which stains completely positive for Vmat-2 has gastric primitivity, whereas a metastasis completely positive for serotonin has an intestinal primitivity. Positive immunostaining for somatostatin and gastrin doesn't identify a specific organ but the anatomic area called "gastrinoma triangle", which includes the distal portion of the stomach, the duodenum and the head of the pancreas; PR and insulin positivity are indicative of a pancreatic primitivity. Particular attention must be paid to PR positivity, because young fertile women can develop pancreatic solid cystic tumors (SCT) which highly resemble pancreatic NETs from the morphological point of view and maintain PR expression, as demonstrated by G. Zamboni *et al.*[5] who reported the immunohistochemical detection of PR in ten cases of SCT. Finally, of particular interest were the observations relative to the tumor-suppressor gene BIN1 obtained in the aforementioned study on gene expression profiles. BIN1 is present in different isoforms with specific tissue distributions and distinct functions. Nuclear BIN1, as found in the prostate and breast, has a tumour suppressor activity due to an ability to activate caspase-independent cell death. In contrast, cytosolic expression of BIN1 is seen in quiescent brain cells. The latter BIN1 isoform has been alternatively named amphiphysin II, given the similarity with the neuronal protein involved in synaptic vesicle endocytosis. Of note, amphiphysins have been described in other neuroendocrine cells, such as enterochromaffin-like cells and adrenocorticotrophin-secreting cells, and seem to play a part in the endocytic processes, but no BIN1 isoforms have been described in the pancreas previously. Validation of our microarray results by means of QRT-PCR and IHC suggests that cytoplasmic forms of BIN1 related to endocytosis are prevalent in islet cells, and that they are strongly overexpressed in PETs. By investigation at the protein level we could detect BIN1 only in the subset of α cells in normal islets, while its expression was extended to the vast majority of tumour cells in some two thirds of PETs. Notably, only one of the 15 non-pancreatic NETs evaluated stained positive for BIN1. This latter finding, together with the higher positivity for BIN1 observed in PET metastases, suggests the role of BIN1 immunostaining as a predictor of a pancreatic origin of distant metastases with neuroendocrine features. The use of organ- and system-specific immunoistochemical markers, such us CDX-2, Vmat-2, serotonin, PR, PP etc., is useful in the detection of unknown primary.

2. Tumor markers in the diagnostic work-up of neuroendocrine tumors

The various cell types of the neuroendocrine (NE) system are characterized by the ability to secrete a variety of bioactive products such as peptides and biogenic amines. Consequently, individual neuroendocrine tumors (NETs) arising from such cells display both a common biological signature related to their neuroendocrine nature as well as a specific biochemical signature unique to the specific molecules they secrete[6]. More specifically, according to their capacity to release bioactive molecules, NETs can be subdivided into two categories: biologically active or functioning NETs,

which produce growth factors, hormones or other local mediators in a constitutive manner and are associated with syndromes related to the hypersecretory activity[7-10], and biologically inactive or nonfunctioning NETs, which exhibit immunopositivity for endocrine markers and/or elevated serum markers but are unassociated with a distinct hyperfunctional clinical syndrome[11]. Over the last two decades, the development of a variety of sensitive and specific plasma and/or serum immunometric assays for the determination of the presence and concentrations of substances produced by these tumors has considerably improved blood-based biochemical diagnosis, thus allowing a more prompt identification of NETs patients and an earlier establishment of surgical and pharmacotherapeutic intervention. From a practical point of view, laboratory biomarkers for the study of gastroenteropancreatic neuroendocrine tumors (GEP NETs) can be subdivided in two major groups: specific tumor markers and non-specific or general tumor markers. Specific markers comprise the individual amines and peptide hormones which are specific to certain NETs histotypes, such as gastrin and basal acid output (BAO) for gastrinoma, insulin and plasma glucose for insulinoma, somatostatin, glucose and chloride for somatostatinoma, vasointestinal peptide (VIP) for VIPoma, glucagon and plasma glucose for glucagonoma, serotonin and its metabolites for carcinoids of the small bowel. General markers include all those molecules that are equally expressed by all NETs and can be used horizontally in all patients to identify NETs in general, such as chromogranin A and neuron-specific enolase (NSE).

2.1 Specific markers: 5-hydroxyindole-3-acetic acid (5-HIAA)

Urinary 5-hydroxyindole-3-acetic acid (5-HIAA) is used in chemical analysis of urine samples as indicator of the body's levels of serotonin. 5-HIAA is the main metabolite of serotonin in the human body and the histotype-specific marker of enterochromaffin (Kultschitzsky) cells (EC). For these reasons, 5-HIAA testing is most frequently performed for the biochemical diagnosis of neuroendocrine carcinomas of the midgut (i.e., duodenum, jejunum-ileum, appendix, right colon), which originate from the EC cells and release great amount of serotonin. This marker has a rather high sensitivity of 70% and a specificity of 90% in patients with tumors producing the typical carcinoid syndrome of flushing, hepatomegaly, diarrhea, bronchospasm and heart disease, which results from hypersecretion of serotonin. However, this marker presents also some limitations. First of all, the determination of urinary excretion of 5-HIAA is not easy since it requires the application of sophisticated methods such as high performance liquid chromatography (HPLC) and, therefore, must be performed only by specialized laboratories. Furthermore, since 5-HIAA is tested by 24-hour urine samples, scrupulous care must be taken that specimen collection and patient preparation have been correct. In fact, certain foods and drugs are known to interfere with the measurement, so it is necessary for patients not to take drugs and avoid food rich in serotonin (bananas, kiwi fruits, avocados, nuts, pineapples, chocolate etc.) for several days before the test. 5-HIAA levels can vary depending on other complications, including tumors, renal malfunction, and small bowel resection. Thus, patients with renal disease may have falsely low urinary 5-HIAA levels, whereas untreated patients with malabsorption, who have increased urinary tryptophan metabolites, may have increased levels of 5-HIAA. Such patients include those with celiac disease, tropical sprue, Whipple disease, stasis syndrome, and cystic fibrosis. Again, 5-HIAA values are increased in patients with chronic intestinal obstruction and may be normal in presence of non metastatic tumors and even of the carcinoid syndrome, particularly in subjects without diarrhea, because some patients with the carcinoid syndrome excrete nonhydroxylated indolic acids.

2.2 General markers: chromogranin A (CgA)

Currently, chromogranin A (CgA) is the best available and most frequently used biomarker for the diagnosis of NETs. CgA belongs to the family of chromogranins and secretogranins, known also as granins, a unique group of acidic, soluble secretory proteins. Granins are ubiquitously distributed within the endocrine, neuroendocrine and nervous systems and are major constituents of the dense-core secretory granules together with peptide hormones, biogenic amines, neurotransmitters, nucleotides and calcium. Several studies have established the presence of granins in a variety of endocrine, neuroendocrine and neuronal tumors, from which they are secreted into the bloodstream. The distributions of granins in neoplasms is generally correlated with their expression in the corresponding normal tissue. The three "classic" granins are chromogranin A, chromogranin B and secretogranin II (sometimes called chromogranin C), and all of them are detectable in plasma or urine samples of NET patients, even if in clinical practice only plasma CgA is used based on its high diagnostic accuracy. CgA is stored and release from the dense-core secretory granules of NE cells along with cell-specific peptides or amines and has essentially two major roles: it is involved in the secretory granulogenesis, secretory protein sorting and secretory granule maturation and condensation; it functions as a sort of prohormone, giving rise to a series of smaller bioactive peptides as a result of post-translational proteolytic processing such as pancreastatin (corresponding to CgA residues 250-301), catestatin (corresponding to CgA residues 352-372) and vasostatin I and II (corresponding to CgA residues 1-76 and 1-115, respectively). These CgA-derived peptide fragments may exert a wide range

of paracrine, endocrine and autocrine functions: they affect secretion of other hormones, play a role in vasoconstriction and regulate metabolism[6,12]. For example, catestatin inhibits catecholamine release from the adrenal medulla, triggers mast cells secretion with consequent release of histamine, promotes chemotactic attraction of monocytes, has a potent antibacterial and antifungal activity against bacteria, fungi and yeast and stimulates polymorphonuclear leukocytes (PMN) secretion[13]. Levels of CgA secretion can be determined in plasma, serum and also in saliva, with salivary CgA which represents a sensitive endocrinological marker of psychological stress. Circulating CgA levels may vary on a day-to-day basis, with a mean daily variability up to 25% of their basal value reported both in healthy subjects and in patients with NETs. Because food intake may increase CgA levels, it is highly recommended to perform CgA measurement in fasting patients. As a rule, levels are considered abnormal when exceeding the upper normal of range by two- to threefold, but the normal range and sensitivity vary according to the assay used. Over the last decades several commercially available radioimmunoassays (RIAs) and enzyme-linked immunosorbent assays (ELISAs) have been developed for the detection of CgA circulating concentrations. These assays vary considerably in their methodology and can produce different results even in the same sample, so it's essential to use always the same assay in the patients' follow-up. For example, the ELISA assay (Dako A/S, Glostrup, Denmark) uses polyclonal rabbit antibodies directed against a 23-kDa carboxyl-terminal fragment of human CgA, and express CgA concentrations in U/L, whereas the IRMA assay (CIS Bio Intern, Gif sur Yvette, France) is based on two monoclonal antibodies directed toward the aminoacid sequences 145-197 and 198-245 and express CgA concentrations in ng/mL. As previously mentioned, CgA has become a standard probe for immunohistochemical analyses of NETs, and elevated levels in blood are diagnostic of these neoplasms. More specifically, increased CgA values are detectable in GEP NETs, pheochromocytomas, neuroblastomas, bronchopulmonary NETs, medullary thyroid carcinoma, paragangliomas, Merkel-cell carcinoma of the skin and other neoplasms.

Bajetta *et al.*[14] evaluated the overall relative sensitivities of the tumor biomarkers CgA, NSE, CEA and 5-HIAA in a large cohort of patients (n = 106) with histopathologically confirmed NETs (including hindgut, midgut, foregut and pancreatic islet cells tumors) referring to the Istituto Nazionale per lo Studio e la Cura dei Tumori in Milan. Among the tested biomarkers, CgA showed the highest overall sensitivity (67.9%) compared to the very low sensitivities observed for 5-HIAA and NSE (35.1% and 32.9%, respectively; Table 1).

Table 2[14] shows marker sensitivity assessed in function of the extension of the disease (i.e., locoregional or metastatic disease). CgA was found to be the most sensitive marker, together with 5-HIAA, in patients with locoregional disease (37.5% for both), and showed the highest sensitivity in patients with lung (80.0%) or liver metastases (78.0%). In comparison, NSE, CEA and 5-HIAA sensitivities in metastatic disease were low or even negligible. Again, CgA proved to be the most sensitive marker for identifying syndromic *versus* nonsyndromic patients (68.4% *vs* 52.8).

The correlation between plasma CgA concentrations and liver involvement was also analyzed in 29 treated patients during a follow-up period of at least 6 months. Notably, increased CgA levels were observed in all patients with numerically or dimensionally augmented liver lesions, compared to stable CgA levels in patients with unchanged lesions and decreased CgA levels in patients with reduced lesions. This study clearly confirms that CgA is the most accurate biomarker available for NETs and provides evidence of its clinical utility not only in the biochemical diagnosis of NETs, but also for identifying patients with locoregional or metastatic spread, particularly to the lung and liver, for discrimi-

Table 1 - Tumor marker specificities and overall sensitivities

		Specificity in patients without any evidence of disease		Overall sensitivity in patients with disease	
Marker	Cut off	Negative/total	%	Positive/total	%
CgA	34.7 U/L	18 of 21	85.7	72 of 106	67.9
NSE	12.5 µg/L	17 of 17	100	29 of 88	32.9
CEA	5.0 µg/L	10 of 11	91.0	10 of 65	15.4
5-HIAA[a]	10.0 mg/L (24 h)	7 of 7	100	13 of 37	35.1

CgA: chromogranin A; NSE: neuron specific enolase; CEA: carcinoembryonic antigen; 5-HIAA: urinary 5-hydroxyindole-3-acetic acid. [a]Marker measurement required only for enterochromaffin-like cell tumors.

Table 2 - Tumor marker sensitivity in patients with disease

Patients (no.)	CgA positive/ total (%)	NSE positive/ total (%)	CEA positive/ total (%)	5-HIAA positive/ total (%)
Locoregional (16)	6 of 16 (37.5)	3 of 13 (23.1)	1 of 10 (10.0)	3 of 8 (37.5)
Metastatic (90)				
Liver	46 of 59 (78.0)	17 of 46 (36.9)	6 of 33 (18.2)	8 of 19 (42.1)
Lung	4 of 5 (80.0)	1 of 5 (20.0)	0 of 3 (0)	0 of 2 (0)
Skeletal	6 of 9 (66.7)	2 of 7 (28.6)	0 of 5 (0)	2 of 3 (66.7)
Multiple	11 of 17 (64.7)	7 of 16 (43.7)	4 of 14 (28.6)	0 of 4 (0)
Syndromic (38)	26 of 38 (68.4)	9 of 30 (30.0)	2 of 24 (8.3)	7 of 12 (58.3)
Nonsyndromic (89)	47 of 89 (52.8)	19 of 75 (25.3)	9 of 52 (17.3)	6 of 31 (19.3)

CgA: chromogranin A; NSE: neuron specific enolase; CEA: carcinoembryonic antigen; 5-HIAA: urinary 5-hydroxyindole-3-acetic acid.

nating between syndromic and nonsyndromic patients and for assessing the response to therapy as well.

The role of CgA as a marker of response to treatment has been evaluated also in a recent study conducted by our Nuclear Medicine Department[15]. In this study, patients with NETs expressing somatostatin receptors were treated with four therapeutic cycles of peptide receptor radionuclide therapy with somatostatin analogs alternating [^{177}Lu]DOTA-TATE and [^{90}Y]DOTA-TATE. Modifications of CgA concentrations compared to baseline were used to evaluate response to treatment. In Figure 2 is shown progressive decrease of serum CgA in responsive patients after each therapeutic cycle[15].

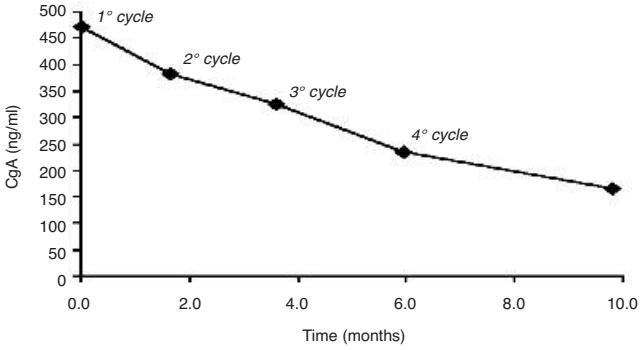

Figure 2 - Reduction of CgA levels in a patient with neuroendocrine carcinoma responsive to 4 cycle of treatment alternating [177Lu]DOTA-TATE and [90Y]DOTA-TATE.

In another retrospective study by Welin S *et al.*[16], patients (n = 56) with radically operated midgut carcinoids were monitored for recurrence 1-3 times per years using plasma CgA, urinary 5-HIAA, radiological examinations and, in a subset of cases, somatostatin receptor scintigraphy and/or positron emission tomography (PET). Notably, elevated CgA proved to be the first indicator of tumor recurrence in the 85% of patients, with level increases evident also in presence of negative ultrasonography, otcreoscan, CT and PET.

Massironi and coworkers have also demonstrated the prognostic value of plasma CgA for identifying patients most likely to be responsive to chronic treatment with somatostatin analogs (SSAs)[17]. In this study, 38 GEP-NET patients received otcreotide 200 µg subcutaneosly and were evaluated for CgA levels at 0, 3 and 6 hours after administration, after which they were given long-term treatment with SSAs. The authors found that patients with a >30% decrease in plasma CgA after otcreotide test were most likely to respond to long-term SSA treatment, confirming the relevant prognostic value of this marker in NETs.

Finally, particular consideration must be paid to possible causes of CgA elevations other than NETs. In fact, although elevations in plasma CgA are mainly associated with the presence of NET disease, several non neoplastic conditions may cause false (non-NET) elevations which, if unrecognized, can lead to misleading interpretations of CgA values with heavy consequences for patients. The main causes of false CgA elevation in clinical practice include hepatic failure and renal failure of all grades, with CgA values increasing in proportion to the degree of renal dysfunction, essential hypertension, inflammatory diseases, chronic atrophic gastritis, situations of chronic stress, the use of acid secretory medications like proton pump inhibitors (PPIs) and H2 receptor antagonists and the use of antihypertensive drugs. Significant rises in CgA have been reported in chronic heart failure as well. CgA is produced by the endrocrine cells of human myocardium and exerts negative inotropic and lusitropic effects on heart. CgA levels correlate with the severity of cardiac dysfunction and are a predictive factor for mortality[6,18-21].

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