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Basics for surgeons about the immunohistochemistry role in pancreatic NETs diagnosis

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Abstract. – OBJECTIVE: Pancreatic neuroendocrine tumors (pNETs) are neuroendocrine tumors primarily found in the pancreas and upper small intestine. There are ten different pNETs: nine of these are associated with a specific functional syndrome, while one is not associated with a specific hormonal syndrome, and it is called non-functional. Up to 90% of pNETs are classified as non-functional. Immunohistochemistry is essential to define the diagnosis. However, to have a correct and reliable diagnosis, the pathologist must have adequately collected and treated tissue samples, thus the surgeon himself should be aware of some fundamental notions about tissue collection and fixation. Although several common biomarkers have been described to date, Chromogranin A and synaptophysin are currently considered the most specific immunohistochemical markers for NETs. Nearly 100% of pNETs are positive for both synaptophysin and Chromogranin A. Therefore, CgA and synaptophysin are effective for well-differentiated NETs but are less helpful in the diagnosis of poorly differentiated NECs, due to dedifferentiation, and then, degranulation of tumor cells. The Neuronal Specific Enolase (NSE) results to be an adequate marker in these cases. Considering the specific markers, many studies reported that endocrine pancreatic neoplasms are able to produce many different polypeptides and amines. Through immunohistochemical techniques, it is possible to define the diagnosis of pNET, which allows the clinicians to direct the patient to an effective therapeutic procedure. But to have a correct and reliable diagnosis, the tissue samples have to be adequately collected and treated.

Key Words:
Pancreatic neuroendocrine tumors, Immunohistochemistry, Chromogranin A, Synaptophysin.

Introduction

Pancreatic neuroendocrine tumors (pNETs) are neuroendocrine tumors found primarily in the pancreas and upper small intestine. pNETs and gastrointestinal carcinoids (GI-NETs) are generally considered separately because of their different syndromes, molecular pathogenesis, biological behaviors, and different response to antitumor treatments[1,2]. Pancreatic neuroendocrine tumors represent less than 5% of all pancreatic tumors[3,4]. There are ten different pNETs (Table I). Nine of these are associated with a specific functional syndrome: gastrinomas (Zollinger-Ellison syndrome), insulinomas, glucagonomas, VIPomas (Verner-Morrison syndrome, pancreatic cholera, WDHA syndrome), GRFomas (growth hormone-releasing factor secreting), ACTHomas, somatostatinomas, pNETs causing carcinoid syndrome and pNETs causing hypercalcemia (PTHrPomas). One pNET is not associated with a specific hormonal syndrome, and it is called non-functional pNET: it is responsible for non-specific symptoms, such as vague abdominal pain, and can be an incidental finding. Up to 90% of pNETs are classified as non-functional[5,6].

Diagnosis

Neuroendocrine tumors (NETs) are defined as "functioning" if they have the ability to produce peptide hormones, causing symptoms that constitute the so-called "carcinoid syndrome". However, the majority of NETs is non-functioning. The traditional laboratory workup comprehends chromogranin A (72-100% sensitivity and 50-80% specificity) and neuron-specific enolase (30-40% sensitivity and up to 100% specificity)[7-10]. If a functional tumor is suspected, the appropriate hormone evaluation has to be performed and followed by computed tomography (CT) or magnetic resonance imaging (MRI). Nuclear imaging with octreotide should be per-

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Table I. Pancreatic neuroendocrine tumor subtypes and syndromes.

	Syndrome	Hormone causing syndrome	Other IIC markers
Functional p-NETs			
Gastrinomas	Zollinger-Ellison syndrome	Gastrin	
Insulinomas		Insulin	
Glucagonomas		Glucagon	Glucagon and its precursors (GLP1, GLP2)
VIPomas	Verner-Morrison syndrome, pancreatic cholera, WDHA syndrome	Vasoactive intestinal peptide	VIP and its precursor pre-pro-VIP (i.e., PHM and cryptic peptide)
GRFomas		Growth hormone releasing factor	
ACTHomas		ACTH	
Somatostatinomas		Somatostatin	
PTHrPomas		PTHrP	
pNETs causing carcinoid syndrome		Serotonin, Tachykinins	
Non-functional p-NETs			Chromogranin A (CgA) Synaptophysin Neuronal Specific Enolase (NSE)

formed to determine if the tumors have a high affinity for somatostatin and if there are occult tumors. Fluorodeoxyglucose positron emission tomography (FDG-PET) is not usually indicated because most pNETs result to be negative[11]; the histological examination is critical for pNET diagnosis, and it could be performed by endoscopic ultrasound (EUS) with a fine-needle biopsy[11]. In particular, immunohistochemistry is essential to define the diagnosis, and Chromogranin A (CgA) and Synaptophysin are currently considered the most specific immunohistochemical markers[12-14].

Immunohistochemistry

The digestive endocrinology, and in particular, the biochemical and clinical characterization of syndromes associated with pNETs, have seen enormous progresses since the 1960s: the great part of the most recent biological and clinical knowledge is due to the application of the immunohistochemical methodologies for disease investigation.

The radioimmunology (RIA) and immunohistochemistry (IIC), frequently used for the diagnostic screening of patients suspicious of endocrine pancreatic neoplasia, allow us to rapidly make the correct diagnosis and to apply an effective therapeutic protocol.

While the RIA is a quantized specific and sensitive investigation to detect peptides and amines in serum, biological liquids or tissue extracts, the IIC is a qualitative investigative technique as it does not measure the quantity of antigen present in endocrine cells. These techniques allow us to formulate a correct diagnosis of nature, site, and biological activity of the neoplasm.

In 1941, Coons et al[10] started to detect the bacterium on a tissue section with immunohistochemical techniques, combining fluorescence with an anti-pneumococcus antibody. In 1955, Coons et al[11] also perfected the technique introducing the indirect immunofluorescence or "sandwich" technique, based on the combination of a species-specific antibody conjugate with fluorescein and direct against the first antibody antigen specific. Among the advantages of this method, there is the greater sensitivity (over 10 times) compared to the direct immunofluorescence method. However, although these methods accurately localized even small antigens, they still had some limitations, such as the rapid decay of the fluorescent properties and the poor ability to study the histological and tissue structures. These limitations resulted consistent in the study of neoplastic tissues.

After that, an enzymatic immunohistochemical system with non-conjugated antibodies was developed, and finally in 1970, Sternberger et

al[12] perfected these techniques by setting the peroxidase-anti-oxidase (PAP) method. With this method, all the problems of conjugation between antibodies and chromogenic substances were overcome, and the sensitivity turned out to be over 100 times compared to the previous methods, thanks to the noticeable amplification in the visualization of the antibody sites. Other progresses were obtained with the production of monoclonal antibodies from hybrid murine cell cultures. This allowed to obtain large amounts of primary antibodies, purified, standardized, and with great properties of specificity and avidity[15].

Immunohistochemical staining techniques can also be used for ultrastructural assessment[16]. However, to have a correct and reliable diagnosis, the pathologist must have adequately collected and treated tissue samples, and so the surgeon himself should be aware of some fundamental notions of tissue collection and fixation.

Concerning the endocrine pancreatic neoplasms, routine fixation in formalin 4% is usually enough, but some cautions have to be observed, i.e., the preparation of tissue samples not bigger than 15-20 mm to allow a correct and homogeneous penetration of the fixative. Other aldehyde fixatives are Bouin, Zamboni, buffered formalin, glutaraldehyde, paraformaldehyde, and some of them are suitable also for further ultrastructural investigations or for *in situ* hybridization.

It is evident that in this phase, a collaboration between pathologist and surgeon is essential, both for the choice of anatomical sites to be biopsied and for the type of fixation to be used, based on the required and adequate investigations.

Non-Specific Markers in pNETS

The histological and cytological aspects of the pancreatic endocrine neoplasms are often typical so as to suggest the diagnosis of endocrine nature, but it is also certain that immunohistochemical techniques are more accurate to provide precise diagnostic elements. In particular, by immunohistochemistry, we can evaluate in neoplastic cells the specific metabolic product (insulin, glucagon, gastrin, etc.) that we will consider specific markers of the neoplasia. These same techniques allow us also to demonstrate the presence of common or non-specific endocrine neoplastic markers.

Although several of these common biomarkers (including CD56, CD57, protein gene product 9.5 PGP9.5, neuron-specific enolase NSE, pan-cytokeratin, and E-cadherin)[17-20] have been described to date, Chromogranin A (CgA) (Figure 1A) and synaptophysin (Figure 1B) are currently considered the most specific immunohistochemical markers for NETs[21-22]. Nearly 100% of pNETs are positive for both synaptophysin and CgA. In particular, while CgA is the most specific marker, synaptophysin is often regarded as the most sensitive one. They are present in the membrane of the intracytoplasmic granules and in the Golgi apparatus of the neuroendocrine cells and of their tumors.

The level of CgA expression may vary according to the degree of tumor differentiation. Well-differentiated NETs are defined by the existence of cytoplasmic neuroendocrine granules and tend to show stronger and more diffuse staining with these neuroendocrine markers than poorly differentiated neuroendocrine carcinomas

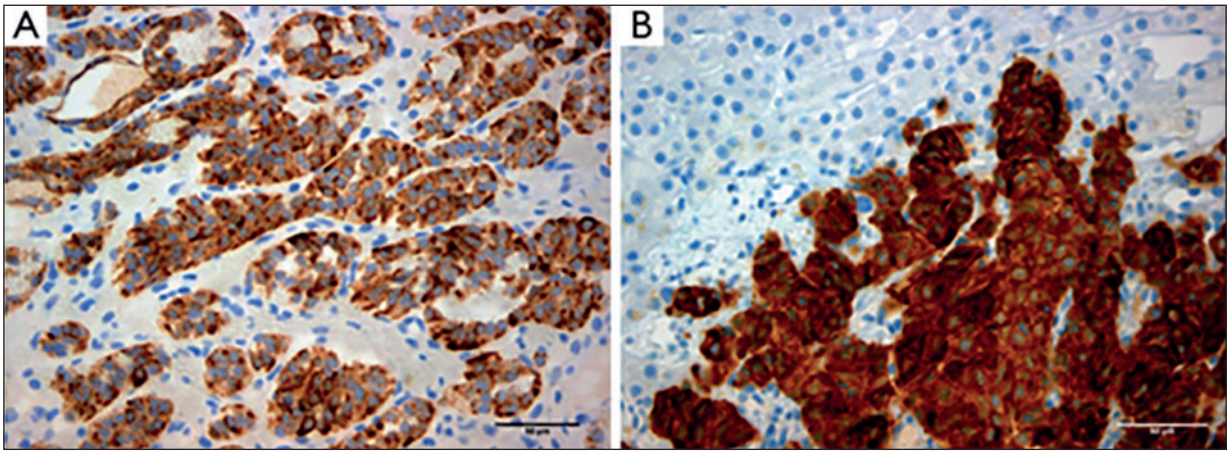

Figure 1. Expression of immunohistochemical markers. Granular cytoplasmic immune expression of chromogranin (**A**) and synaptophysin (**B**). (100× OM).

(NECs)[23]. Poorly differentiated NECs are further classified as small cell carcinomas and large cell carcinomas. In large cell NECs, a positivity for synaptophysin is mandatory, while CgA staining is variable and may be weak or absent[24].

Therefore, CgA and synaptophysin are effective for well-differentiated NETs, but are less helpful in the diagnosis of poorly differentiated NECs, due to dedifferentiation, and then, degranulation of tumor cells. The Neuronal Specific Enolase (NSE) results to be an adequate marker in these cases. It is a dimer of the enzyme Enolase, present in all eukaryotic cells[25]. It was first located in the neuronal cells of the central system, and therefore also in other cell types, such as the neuroendocrine cells of the gastroenteropancreatic system and in its neoplasms[26,27].

NSE got a bad reputation because it was found not only in NETs but also in tumors classified as adenocarcinomas or undifferentiated carcinomas and its specificity was therefore considered limited. But it resulted useful and effective in the case of neuroendocrine neoplasms with few or no intracytoplasmic granules[6].

The others non-specific biomarkers are not recommended in the clinical practice because of their low specificity: neural cell-adhesion molecule (NCAM) or CD56 is a membrane-bound glycoprotein involved in neuron-neuron and nerve-muscle interactions. It is sensitive but not highly specific because it is often expressed in several non-NETs. It seems to be expressed in only 44% of pNETs[5]. Caudal type homeobox 2 (CDX2) has been detected in tumors originating from some parts of the gastrointestinal tract, whereas some pNETs also express this marker. Several transcription factor proteins, such as pancreatic and duodenal homeobox 1 (PDX1), islet 1 (ISL-1), have been reported to be pancreas-specific[27]. Paired box genes 6/8 (PAX 6/8) can be expressed in the islets of Langerhans, suggesting the pancreatic origin of NETs. However, more recent studies have reported that monoclonal PAX8 antibodies are universally non-reactive with these genes.

Specific Markers in pNETS

Well-differentiated pNETs can be classified into functioning and non-functioning tumors based on the clinical symptoms induced by hormonal hypersecretion. About one-half of pNETs are functioning tumors, and insulinomas are the most common, followed, in order of frequency, by glucagonomas, gastrinomas, and somatostatinomas.

Considering the specific markers, many studies reported that endocrine pancreatic neoplasms are able to produce many different polypeptides and amines thanks both to the presence of different cellular lines and to the ability of these cells to have more than one product[27]. Finally, these substances are often produced in different molecular forms such as pro-peptides with a high molecular weight that can be stored in the cell cytoplasm or cleaved and placed in blood circulation. Therefore, it is very important to have primary antibodies able to identify not only antibodies for the polypeptides responsible for the endocrine syndrome associated with the neoplasia, but also for the aminoacidic sequences extended to terminal N and C of the polypeptide (pre-propeptide). If we consider the pancreatic glucagon molecule and its precursor (pre-proglucagon), we can observe that the active biologic peptide of 28 amino acids is joined by constant sequences at both N and T terminals (GLP1, GLP2, Glicentin).

Examining 10 cases of pancreatic glucagonomas, Bishop et al[25] have clearly shown that these molecular forms (preglucagon) are always present in neoplastic cells and that the combined use of different antibodies for pancreatic glucagon and its precursors allowed a better morphologic and functional characterization of neoplasms. According to their study, it was not possible to observe immunoreactivity for pancreatic glucagon in three cases but only for some sequences of its precursors (GLP1, GLP2).

Similar findings were obtained considering the immunoreactivity of the neoplastic cells of 8 VIPomas. Having in fact antibodies against VIP and against some sequences of its precursor pre-pro-VIP (i.e., PHM and cryptic peptide), it has been possible to demonstrate the presence of these peptides in many of the observed cases, even in the case in which VIP was not found to be immunoreactive[27].

Neuroendocrine euplastic cells of the gastroenteropancreatic system have been clearly shown to be able to produce two or more regulators peptides, and so it is accepted that these cells can contain one or more neuroendocrine mediators or could differentiate in different lines with different metabolic activity. It is frequent the presence of some cellular types (i.e., pp cells) in some neuroendocrine pancreatic tumors with different primary metabolic activity, such as gastrinomas, glucagonomas, etc.[27]. For this reason, the pancreatic polypeptide (PP) has been listed among endocrine pancreatic tumors markers.

However, it is important to underline that a pancreatic neuroendocrine neoplasm with elevated metabolic activity could sometimes result negative for the immunohistochemical research of peptides considered associated to the syndrome, because secretive cellular processes do not allow accumulation of these substances. For example, we could have gastrinoma not very reactive with antigastrin-antibodies, but equally responsive to other polypeptides of the neuroendocrine system. Another possibility is the low secretive activity of the tumor, with high intracellular accumulation and low circulation levels of itself, and so with an absent or attenuated endocrine syndrome[27].

The systemic endocrine symptoms could also be absent when the neoplasm does not produce mediators in forms that are biologically active or when there is a wrong receptor mechanism or when the neoplasm produces mediators with mutually antagonistic action. We also have to consider that the same antibody can give different results depending on the immunohistochemical technique used. An antibody with excellent response in radioimmunology or immunodiffusion could not be adequate to immunohistochemistry because it does not act in a solution or in a homogeneous environment, but it acts on a tissue section with different antigens that could be altered by fissation, inclusion etc. Therefore, the clinicians do not have to be surprised if there are significant discrepancies between syndrome, hematic levels of the peptide, and immunohistochemical reports[25-27].

From the beginning of the 1980s to the present we have seen a big progress of the methods to assess the nuclei acids, so today we are able to know the exact genome sequence for many proteins' synthesis, for example for neuroendocrine peptides and their precursors. *In situ* hybridization techniques allow us to identify intracellular messenger-RNA coded for the synthesis of a specific polypeptides, and we can measure its quantity.

Therefore, with these techniques we are able to determine the neuroendocrine nature of a pancreatic neoplasm also with a low grade of antigenic intracellular accumulation, but a more adequate discussion of these methods is beyond the scope of our paper.

Conclusions

Through immunohistochemical techniques, it is possible to exactly define the diagnosis of pNET, and this allows the clinicians to direct the patient to an effective therapeutic procedure. But in order to have a correct and reliable diagnosis, the tissue samples have to be adequately collected and treated. In this perspective, it is essential in this phase a collaboration between pathologists and surgeons, which implies that the surgeon should be aware of some fundamental notions about the immunohistochemical techniques.

Conflict of Interest
The Authors declare that they have no conflict of interests.

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