

Role of immunohistochemistry in the diagnosis of lung cancer*

Papel da imuno-histoquímica no diagnóstico do câncer de pulmão

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Abstract

The role of immunohistochemistry is to recognize antigens and, consequently, to identify and classify specific cells within a cell population whose morphology is heterogenous or apparently homogenous. The visualization of the antigen-antibody complex is made possible through the addition of either a fluorochrome conjugate or an enzyme to the antibody, which is then viewed under microscopy. Immunohistochemistry can be used in the routine diagnosis of lung cancer, in order to identify biological markers (diagnostic and prognostic). The essential immunohistochemistry panels will be discussed in this review.

Keywords: Immunohistochemistry; Tumor markers, biological; Lung neoplasms.

Resumo

O propósito da imuno-histoquímica é reconhecer antígenos e assim identificar e classificar células específicas dentro de uma população celular morfológicamente heterogênea (ou aparentemente homogênea). A visualização do complexo antígeno-anticorpo é possível pela adição de um fluorocromo conjugado ao anticorpo, que pode então ser observado ao microscópio, ou alternativamente uma enzima, cujo produto de reação pode igualmente ser visualizado. A imuno-histoquímica pode ser aplicada na rotina diagnóstica complementar do câncer de pulmão para a identificação de marcadores biológicos diagnósticos e prognósticos. Os painéis imuno-histoquímicos mínimos necessários para a complementação diagnóstica serão discutidos nesta revisão.

Descritores: Imunoistoquímica; Marcadores biológicos de tumor; Neoplasias pulmonares.

Classification

Carcinomas account for nearly 95% of all cases of lung cancer, whereas sarcomas and lymphomas account for most of the remainder. Clinically, lung carcinomas are classified as non-small cell lung cancer (NSCLC) or small cell lung cancer. According to the current World Health Organization classification,⁽¹⁾ NSCLC includes the following histological types: squamous cell carcinoma; adenocarcinoma; and large cell carcinoma. In the same classification, small cell carcinomas are considered neuroendocrine carcinomas, as are the following histological types: typical carcinoid tumor, atypical carcinoid tumor and large cell neuroendocrine carcinoma. Accounting for 75-80% of all cases, NSCLC is the more common of the two types.⁽²⁾ Clinical staging and identification of the histological type are fundamental to establishing the therapeutic strategy for treating such patients. In earlier stages, surgical resection offers a real

chance of cure.⁽³⁾ However, even in clinical stage IA (T1N0M0), 30-40% of patients will die as a consequence of the progression of the cancer,⁽⁴⁻⁶⁾ especially due to systemic recurrence. Therefore, the identification of the different histological types and of prognostic factors is important and has the purpose of selecting patients for receiving appropriate treatment, in addition to identifying tumors at higher risk of recurrence. These selected patients would potentially benefit from therapeutic approaches directed at the histological type, as well as from adjuvant therapies aimed at avoiding cancer recurrence and fatal outcomes. The intention of such classification systems is to provide light microscopy criteria that allow pathologists to establish consistent diagnoses among themselves. With such discipline, comparatively, treatment protocols can then be established. When the criteria proposed by these classification systems are observed,

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the diagnostic reliability among experienced pathologists is 90%.⁽¹⁾ The high inter-rater variability is found in the identification of large cell carcinoma in cases of squamous cell carcinoma and poorly differentiated adenocarcinoma. In this context, immunohistochemistry represents a tool that can provide a clear distinction among the various types.

Immunohistochemistry

The objective of performing immunohistochemistry is to recognize cell constituents (antigens) and, consequently, to identify and classify specific cells within a cell population whose morphology is heterogenous or apparently homogenous. The visualization of the antigen-antibody complex is made possible through the addition of either a fluorochrome conjugate or an enzyme to the antibody, which is then viewed under microscopy. In some cases, the antibodies are not entirely specific and can cross-react with more than one antigen, or there can be false-positive and false-negative results, which are caused by high levels of background staining and absorption. In addition, some cells can express antigens typically associated with completely different types, such as prostate-specific antigen in the pancreas or lymphoid antigens in lung tumors. Therefore, appropriate sections of control tissues (such as lymph nodes or tonsils for lymphoid antigens) are always processed together with lung sections, and these controls are always checked before the evaluation of a lung tumor.

Immunohistochemistry can be used in the routine diagnosis of lung cancer in order to identify biological markers (diagnostic and prognostic). Most pathological anatomy laboratories have the essential immunohistochemistry panels necessary to complement the diagnosis.

Figure 1 illustrates an immunohistochemistry panel for epithelial markers (cytokeratin AE1/AE3 and cytokeratin 7–CK7), neuroendocrine markers (chromogranin, synaptophysin, thyroid transcription factor-1 [TTF-1] and p53) and mesenchymal markers (leukocyte common antigen and smooth muscle actin).

Biological diagnostic markers

Lung biopsy is one diagnostic approach to lung cancer. Its primary intent is to classify

tumors as one of the four major histological types: squamous cell carcinoma, adenocarcinoma, neuroendocrine carcinoma and large cell carcinoma. When technical problems related to sampling, artifactual problems or problems related to undifferentiated cells do not allow a definitive classification, every effort should be made to identify, at least, the histogenesis (carcinoma vs. sarcoma vs. lymphoma) and separate undifferentiated large cell tumors from undifferentiated small cell tumors. It is true that, in some situations, it is not possible to make even these broad categorizations, due to lack of analyzable material. In this context, immunohistochemistry is an indispensable complementary tool, as we will here discuss in detail.

Although the reliability rate for neuroendocrine carcinoma, especially small cell carcinoma, is approximately 85%, in 15% of the cases, it is necessary to perform an immunohistochemistry panel including the following markers^(1,7): high- and low-molecular-weight cytokeratins (AE1/AE3, Figure 1a); cytokeratins specific for primary pulmonary origin (CK7, Figure 1b); cytokeratins for primary gastrointestinal tract origin (CK20); markers of neuroendocrine differentiation (chromogranin, synaptophysin and CD56; Figures 1c and 1d); TTF-1 (Figure 1e); p53 (Figure 1f); and markers of lymphoid origin (leukocyte common antigen; Figure 1g). markers of mesenchymal origin (vimentin and smooth muscle actin; Figure 1h); The use of CK7 and CK 20 also offers the opportunity to establish whether the tumor is a primary pulmonary lesion (CK7+ and CK20–) or a metastatic lesion of the digestive tract (CK7– and CK20+).

Neuroendocrine differentiation

Once a diagnosis of neuroendocrine tumor has been established, it is necessary to classify the tumor as a high-grade neuroendocrine tumor (small cell carcinoma or large cell neuroendocrine carcinoma) or a low-grade neuroendocrine tumor (typical carcinoid tumor or atypical carcinoid tumor).⁽⁷⁾

Typical carcinoid tumor or low-grade neuroendocrine carcinoma

Typical carcinoid tumor or low-grade neuroendocrine carcinoma is characterized by 0 or 1 mitoses per 2 mm², absence of necrosis,

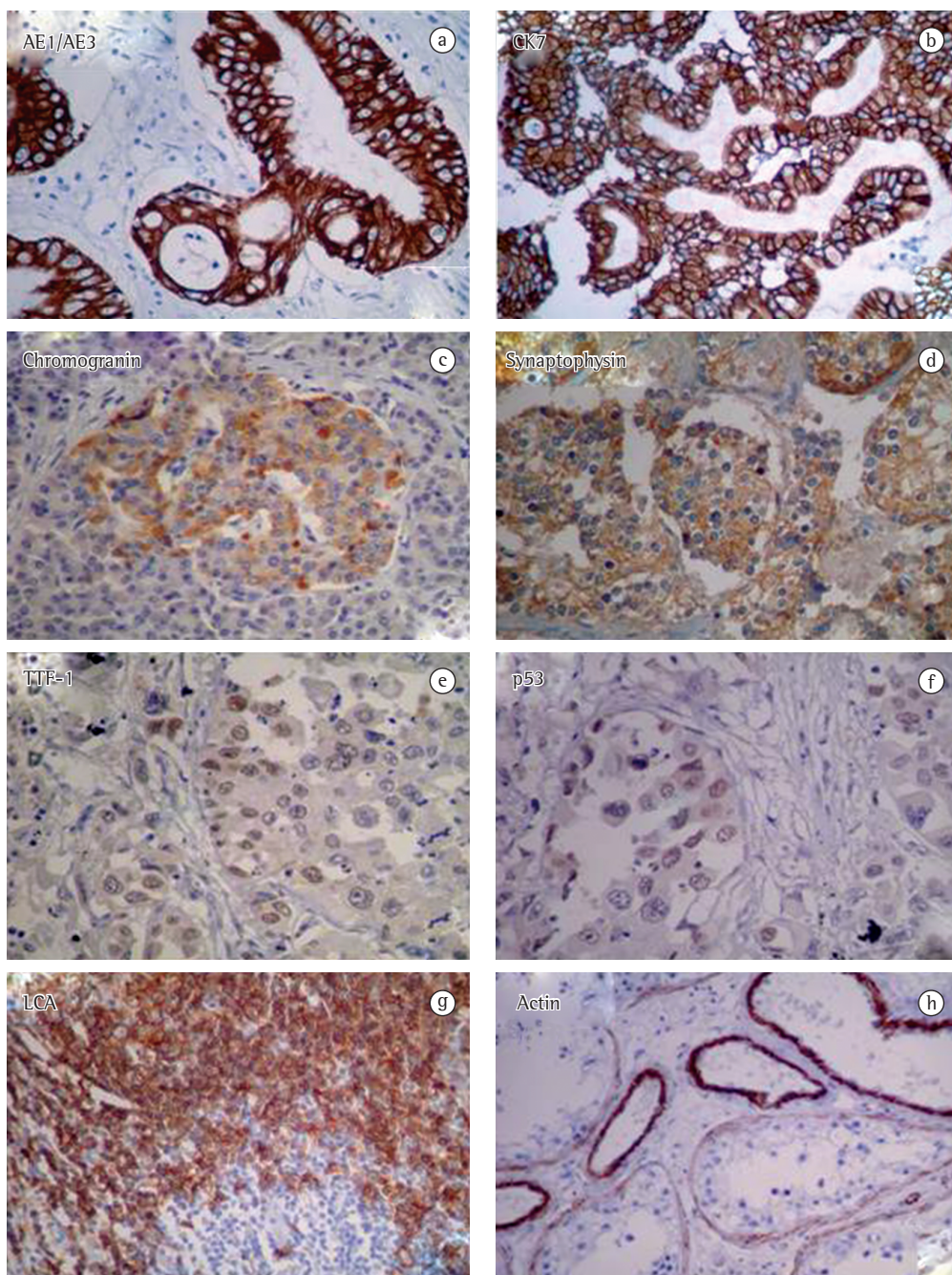


Figure 1 - Immunohistochemistry panel used in the routine diagnosis of lung cancer. Positive immunohistochemistry reaction is revealed by brown staining in the nuclei (thyroid transcription factor-1–TTF-1–and p53) or cytoplasm (cytokeratins, smooth muscle actin and leukocyte common antigen–LCA). In a), an adenocarcinoma whose neoplastic cells strongly express the high- and low-molecular-weight cytokeratins AE1 and AE3, revealed by the strong brown staining in the cytoplasm. In b), an adenocarcinoma with strong immunohistochemical expression for the low-molecular-weight cytokeratin 7 (CK7), whose presence also favors the primary pulmonary origin of the tumor. Neuroendocrine markers of chromogranin (in c) and synaptophysin (in d) are identified by the brown staining in the cytoplasm of the neoplastic cells of a typical carcinoid tumor. Expression of markers related to nuclear activity (TTF-1) and apoptosis (p53), revealed by the brown staining in the nuclei, is illustrated in e) and f), in a primary lung adenocarcinoma. The mesenchymal origin of a neoplasm can be revealed by CD20 antigen expression (CD20 lymphocytes) in the case of a lymphoma (LCA, in g) or by smooth muscle actin expression in the blood vessel walls (in h). Immunohistochemistry, $\times 400$ (A-F).

neuroendocrine structures (rosettes, trabeculae and solid nests) and chromogranin expression in the cytoplasm.

Atypical carcinoid tumor or low-grade neuroendocrine carcinoma

Atypical carcinoid tumor or low-grade neuroendocrine carcinoma is defined by 2-10 mitoses per 2 mm², necrosis, neuroendocrine structures (rosettes, trabeculae and solid nests) and chromogranin expression in the cytoplasm.

Large cell neuroendocrine carcinoma

Large cell neuroendocrine carcinoma is defined by numerous mitoses (typically more than 30 per 2 mm²); large nuclei (25-35 µm); an organoid structure, similar to that of a carcinoid tumor; extensive areas of necrosis; and positivity (in more than 25% of the cells) for chromogranin, synaptophysin and other neuroendocrine markers.

Small cell neuroendocrine carcinoma

Small cell neuroendocrine carcinoma is defined by numerous mitoses, an organoid structure, a nuclei between 16-23 µm, dense heterochromatin, invisible nucleoli, an invisible or scarce cytoplasm and positivity for the neuroendocrine markers chromogranin, synaptophysin and TTF-1.

Non-neuroendocrine differentiation

Non-small cell carcinoma

In view of the therapeutic options currently available, if the tumor is non-neuroendocrine, every effort should be made to separate, at least, small cell lung cancer from NSCLC, using light microscopy. It should be borne in mind that squamous cell carcinomas, adenocarcinomas and large cell carcinomas without evidence of differentiation in routine H&E staining are included under the designation NSCLC. In this context, stainings designed specifically for histochemistry (mucin) and immunohistochemistry should be considered. When NSCLC is suspected of being adenocarcinoma, the first step is to determine whether it is primary or metastatic using the following panel: primary lung tumor (TTF-1+

and CK7+); primary digestive tract tumor (CK20+); primary thyroid tumor (positive for thyroglobulin); primary prostate tumor (positive for prostate-specific antigen-PSA); primary breast tumor (positive for estrogen receptors, progesterone receptors and Br-est); and primary ovarian tumor (CA125+). Primary or metastatic squamous cell carcinomas of the lung are usually positive for high-molecular weight cytokeratin 34βE12, cytokeratin 5/6 (CK5/CK6) and low-molecular weight cytokeratin 35βH11—few cases are TTF-1+ and CK7+.⁽¹⁾ To date, no immunohistochemistry markers to differentiate primary carcinomas from metastatic carcinomas have been identified. One group of authors demonstrated the importance of p63 protein expression, as well as of CK5/CK6 and CK7 expression and surfactant protein A (SP-A) expression, in the classification of 42 types of NSCLC in autopsy and surgical resection specimens.⁽⁸⁾ The authors also showed the usefulness of these markers in differentiating squamous cell carcinomas from adenocarcinomas based on the implications for prognosis and treatment. All adenocarcinomas were p63-, 9/16 (56.2%) were CK5/CK6+, 16/17 (94.1%) were CK7+, and 4/15 (26.6%) were SP-A+. Of the squamous cell carcinomas, 1 case was CK7+ and SP-A+, and 14/18 (77.8%) were p63+. The authors concluded that p63 protein expression is useful in differentiating between the two histological types.

Bronchioloalveolar carcinoma

Among the various histological subtypes of adenocarcinoma, the one that should be prioritized, in terms of identification, is bronchioloalveolar carcinoma, due to its prognostic implications. The most significant histological characteristic of bronchioloalveolar carcinoma is growth along the alveolar walls, without destruction of the lung architecture. Although histological recognition of bronchioloalveolar carcinoma does not require the use of complementary techniques, immunohistochemical studies typically reveal expression of TTF-1 and SP-A.

Biological prognostic markers

Immunohistochemistry can be used in the routine diagnosis of bronchogenic carcinoma in order to identify biological prognostic markers.

Although there is no unanimous panel to detect lung cancer in our diagnostic routine, we have been using p53, c-ERB, vascular endothelial growth factor (VEGF), cyclooxygenase (COX) and matrix metalloproteinase 9 (MMP-9).

p53

Mutations in the *p53* gene might not produce the p53 protein or might produce dysfunctional p53. The mutant p53 protein lives much longer than does the wild-type p53 protein, resulting in high p53 protein levels in transformed cells. The *p53* gene is located on chromosome p17. Abnormalities in the *p53* gene are common in lung cancer, usually as a point mutation. The encoding protein (p53) is probably a nuclear transcription factor and is a tumor suppressor factor acting through mechanisms that have yet to be elucidated. The *p53* gene regulates cell growth at the G1-S interface of the cell cycle and plays an important role in inducing apoptosis of cells with damaged DNA. Mutations in the *p53* gene seem to be associated with exposure to environmental carcinogens, such as cigarette smoke.⁽⁹⁾ Although abnormalities in *p53* gene expression do not seem to be associated with prognosis in mixed lung tumors, they worsen prognosis in patients with stage I adenocarcinoma.⁽¹⁰⁾ In animal models, it has been demonstrated that the wild-type *p53* gene can suffer transduction into round lung cancer tumor cells with a retroviral vector. If the tumor cells are homozygous for a mutant *p53* gene, there will not be significant growth inhibition after transduction, nor will there be wild-type *p53* expression, with induced apoptosis of round tumor cells. This raises the possibility of a new therapeutic strategy in lung cancer presenting the *p53* gene mutation. One group of authors⁽¹⁰⁾ studied surgically resected primary lung adenocarcinomas and demonstrated that staging based on the size of the neoplasm and on the neoplastic involvement of the lymph nodes is extremely useful in estimating the biological behavior of this neoplasm, as are patient gender, degree of stromal proliferation and immunohistochemistry detection of p53 protein in neoplastic cells. These factors could be used as prognostic markers of disease progression. Another study⁽¹¹⁾ demonstrated that early stages, as well as low expression of argyrophilic nucleolar organizer region (AgNOR), low p53 expression and high Bcl-2 expression, had a

positive impact on survival among patients who underwent surgery. The authors also found that lymph node involvement was strongly associated with high p53 and AgNOR expression, as well as that p53 and AgNOR are major parameters in determining prognosis in such patients. More recently,⁽¹²⁾ it was demonstrated that immunohistochemical expression of p53 correlates with lower survival but not with gene mutations in resected tumors.

c-erbB-1

The c-erbB-1 proto-oncogene, when linked to the membrane, encodes a growth receptor (170-kDa tyrosine kinase) that is the receptor for the epidermal growth factor receptor (EGFR). In the normal lung, c-erbB-1, through its protein product, acts to stimulate epithelial cell proliferation and to promote airway maturation during embryonic development. This proto-oncogene has been shown to be overexpressed in NSCLC, especially in squamous cell carcinomas (in 65-90% of the cases reported). The use of c-erbB-1 gene overexpression as a marker for clinical prognosis is controversial, some studies showing an association with low survival and others showing no such association.

c-erbB-2

The c-erbB-2 proto-oncogene belongs to the c-erbB family of receptors linked to the tyrosine kinase membrane, it is structurally related to the c-erbB-1 proto-oncogene. The protein that encodes c-erbB-2 is known as p185c-erbB-2, or HER2. This protein is expressed in the epithelial cells of the airways of the normal lung, playing a role in the growth and differentiation of the normal lung epithelium. It has been observed that HER2 is produced in conjunction with EGFR in many lung adenocarcinomas. One group of authors⁽¹³⁾ recently demonstrated that survival is strongly associated with cyclin D1, c-erbB-2 and VEGF expression in resected primary adenocarcinomas and their metastases.

MMP-9

Before there are metastases, a cell or a group of cells must detach themselves from the primary tumor, penetrate the adjacent tissue, survive and grow in the host tissue. Squamous cell carcinoma is the only known histological subtype

that progresses through an in situ stage to invasive carcinoma. This requires interaction with the subepithelial basal membrane and involves three steps: 1) adherence to the subepithelial membrane; 2) creation of a defect in the basal membrane; and 3) translocation of the tumor cells through the basal membrane. Tumor cell adherence involves the binding between tumor cell surface proteins and glycoproteins, such as laminin, collagen IV and fibronectin. These bindings are influenced by various adhesion molecules, including integrins, which represent a family of transmembrane glycoproteins functioning as adhesion receptors. The tumor cells form a cleft in the basal membrane by producing hydrolytic enzymes or by stimulating the host cells to produce proteinases. Examples of proteolytic enzymes include metalloproteinases, collagenases IV, serum proteases (such as urokinase) and cysteine proteases (such as cathepsins B and L). Translocation through clefts in the basal membrane is directed by chemotactic substances contained in the host cells or by motility factors produced by the tumor cells. One group of authors⁽¹⁴⁾ demonstrated the importance of proteases in the process of tumor invasion; MMP-9 expression in surgically resected primary lung adenocarcinomas confirms the potential for the use of metalloproteinase inhibitors in the treatment of these neoplasms. In another elegant study,⁽¹⁵⁾ the results obtained suggested that staining for COX-2 and for MMP-9, as well as tumor categorization into papillary and nonpapillary, can provide important prognostic information to patients with resected lung adenocarcinomas; it is possible that all three variables can inform decisions regarding post-operative adjuvant treatment. Another group of authors⁽¹⁶⁾ recently demonstrated that TTF-1 and MMP-9 expression by immunohistochemistry allows the identification of different prognostic groups among patients with adenocarcinomas treated with platin. In the same line of investigation, one study⁽¹⁷⁾ had already demonstrated that expression of markers related to apoptosis and to the tumor microenvironment, such as Bcl-2 ($> 3.1\%$) and extracellular matrix density ($< 9.8 \text{ mm}^2$), includes a subgroup at high risk for lymph node metastases and can be an appropriate target for prospective adjuvant studies after surgical resection. More recently, there was a multicenter study involving 330 patients

submitted to surgery due to bronchial carcinoma tumor and monitored for more 10 years in two university hospitals.⁽¹⁸⁾ The authors used the following data to predict the individual risk for node metastases or distant metastases: age; gender; tumor-node-metastasis staging; tumor diameter; tumor location (central or peripheral); immunohistochemical index for p53, Ki67 and Bcl-2; and density of newly formed microvessels and fibers of the collagen-elastic system. The incidence of lymph node metastases and distant metastases was 11% and 5%, respectively. The univariate analysis associated biomolecular markers with node metastases. The multivariate analysis identified neoangiogenesis as a predictive marker for node metastasis. Other important interactions were demonstrated by one group of authors⁽¹⁹⁾ who, while studying how the characteristics of the tumor cell relate to stroma for proliferative factors, p53, vascular microdensity and metalloproteinases, controlled for extent of the primary lesion (T1 to T4), in limited (non-metastatic) NSCLC and advanced (metastatic) NSCLC, suggested that different interactions between the tumor cells and the stromal cells control the metastases and, therefore, control the biological risk for NSCLC.

VEGF

Angiogenesis represents an important factor since it allows tumor growth, tumor invasion and metastases. The genes or molecules that participate in tumor invasion and metastases are regulating factors that have a positive or negative character. The VEGF promotes the formation of blood vessels at the beginning of the development (vasculogenesis) and plays a central role in the growth of new blood vessels (angiogenesis). Its role in tumor growth is fundamental, since, without vascularization, tumors cannot exceed 1-2 mm in diameter or in thickness. In addition, angiogenesis is indispensable for the formation of metastases. Two of the greatest inducers of angiogenesis produced in lung cancer are VEGF and fibroblast growth factor.⁽²⁰⁾ There have been various studies showing the relationship between high VEGF levels and poor prognosis in cases of NSCLC.^(13,20) One group of authors⁽²¹⁾ recently demonstrated the importance of VEGF as a prognostic marker in adenocarcinomas and their hematogenous metastases.

Final considerations

Immunohistochemistry represents an important complementary tool for the routine diagnosis of lung cancer and for the identification of the different histological types and prognostic factors. Its purpose is to categorize patients in order to ensure appropriate and specific treatment, as well as to identify tumors at higher risk of recurrence and fatal outcomes. The essential immunohistochemistry panel recommended for the diagnosis and prognosis includes expression of the following markers: CK7; CK20; COX-2; TTF-1, chromogranin; synaptophysin, CD56; PSA; CA125; p53; c-erbB-2; MMP-9; and VEGF.

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