9 Immunohistochemistry and Molecular Biology in Transbronchial Cryobiopsies

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9.1 Introduction

Invasive procedures are frequently needed in the diagnostic workflow of pulmonary pathology, in both neoplastic and nonneoplastic cases. Precise diagnoses, complete of all molecular and immunophenotypic data, are requested by updated WHO tumor classifications and precision-medicine criteria, but this need has to be reconciled with the emerging requests of minimally invasive methods for obtaining tissue samples aimed to minimize patients' risks and discomfort [1]. In this scenario, pathologists are faced by the contradictory demands for maximal data and minimal tissue availability. The morphological (histological) analysis of a tissue sample still remains the most informative and economic diagnostic tool, usually performed on hematoxylin- and eosin-stained slides (H&E), and this approach still represents the first step in pulmonary pathology. Nevertheless, morphology alone is not sufficient to provide all the diagnostic, prognostic, and predictive information needed by updated protocols in pulmonary oncology.

Immunohistochemistry represents a widely utilized method to provide relevant information on the antigenic/proteomic profile of atypical cells, allowing the precise definition of the "cell of origin" of a tumor (e.g., squamous cell carcinoma versus adenocarcinoma, poorly differentiated lung carcinoma versus epithelioid mesothelioma, etc.), and also details on the expression of molecules with prognostic and/or predictive significance, such as ALK, ROS, PDL1, p53, and others [2–7] (Table 9.1). Molecular tests (EGFR mutation status and others) are also necessary in the managing of oncologic patients, and the availability of these tests is nowadays a major issue when either cytological samples or small biopsies are only accessible [1, 8–12].

Diffuse parenchymal lung diseases (DPLD) are a wide group of disorders with heterogeneous clinical presentation, prognosis, and pathogenesis. The diagnosis of these diseases is generally obtained by the coordinated evaluation of labora-

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Table 9.1 Suggested immunohistochemical markers in lung pathology

Pathology	Suggested immunostains
Lung carcinoma (general)	CK7, CK5/6, ΔN-p63, MUC5AC, TTF1, Napsin-A, CDX2, CD56, Synaptophysin, ALK, p53, *others*
Squamous	CK5/6, ΔN-p63, p53
Adenocarcinoma	CK7, TTF1, Napsin-A, MUC5AC, CDX2, p53
Small cell	CD56, Synaptophysin, Chromogranin, p53, CK7
Mesothelioma	Calretinin, WT1, Podoplanin, CK5/6, BerEP4, BAP1
Lymphoma	CD20, CD3, EBV/EBER, CD30, kappa, lambda, granzyme, p53, *others*
Langerhans' cell hystiocytosis	CD1a, S100, CD68, Langerin, BRAF
Lymphangioleyomiomatosis	HMB45, α-SMA, Cathepsin-K
Erdheim-Chester disease	CD68, S100, BRAF
Infections	EBV/EBER, HHV8, Pneumocystis J, CMV, *others*
Hypersensitivity pneumonitis	Cathepsin-K
PPFE	Elastin, Podoplanin
IPF and other ILD	CK8/18, CK5/6, Tenascin, α-SMA, hsp27, Laminin-5-γ-2, β-catenin, p16, p21

tory, imaging, and clinical data, but frequently a histological evaluation on lung biopsies is needed to substantiate the diagnosis. Large surgical biopsies (SLB, generally obtained by VATS—video-assisted thoracic surgery) are usually considered the gold standard for histological examination, but the risks related to this procedure have suggested that alternative, less invasive, techniques should be performed [13, 14].

Transbronchial lung biopsies, especially transbronchial cryobiopsies (TCB), have been recently described as an alternative to SLB, since they allow a good sensitivity with minor risks and costs [15–17]. The clinical applications of TCB are increasing, with experiences available in the diagnosis and characterization of endobronchial tumor lesions [18–20], pleural lesions [21, 22], infections [20], and DPLD [15, 17, 23, 24]. Nevertheless, since TCB provide less tissue for morphological analysis than SLB and about 20% TCB are nondiagnostic [24], some concerns have been recently raised on the opportunity to change [25]. In a series of previous publications, we have suggested that the diagnostic sensitivity and specificity on transbronchial biopsies can be significantly increased by utilizing a limited number of immunohistochemical markers related to the different pathogenesis of DPLD [26–28]. This approach, which can be also applied to TCB, may potentially increase the yield of morphologic analysis, thus reconciling the need of both more safe procedures and high sensitivity [29].

According to different studies, immunohistochemical markers can be successfully demonstrated on TCB, as can be molecular tests carried out on nucleic acids extracted from cryobiopsies [16, 29], without any specific problem related to this new procedure (Fig. 9.1). The minimal artifacts that can at times be observed on TCB [30] do not affect the staining quality of all tested nuclear-, cytoplasmic-, and membrane-located antigens [29].

9.2 Immunohistochemistry in Neoplastic Pulmonary Pathology

Immunohistochemistry is a non-expensive methodology that is widely available in most histological laboratories and is broadly applied in the characterization and diagnosis of tumors according to WHO guidelines. Specific immunophenotypic profiles are available for different types of human malignancies (e.g., lymphomas and leukemias, lung carcinoma, breast carcinoma, renal cell carcinoma, etc.). In lung carcinoma, well-established sets of immunohistochemical markers have been introduced in clinical practice that allow the precise characterization of tumor cell differentiation (e.g., adenocarcinoma versus squamous cell carcinoma, versus small-cell carcinoma), as well as markers helping in the differential diagnosis between primary lung carcinomas and either mesothelioma or metastatic spread from other sites [2, 31, 32].

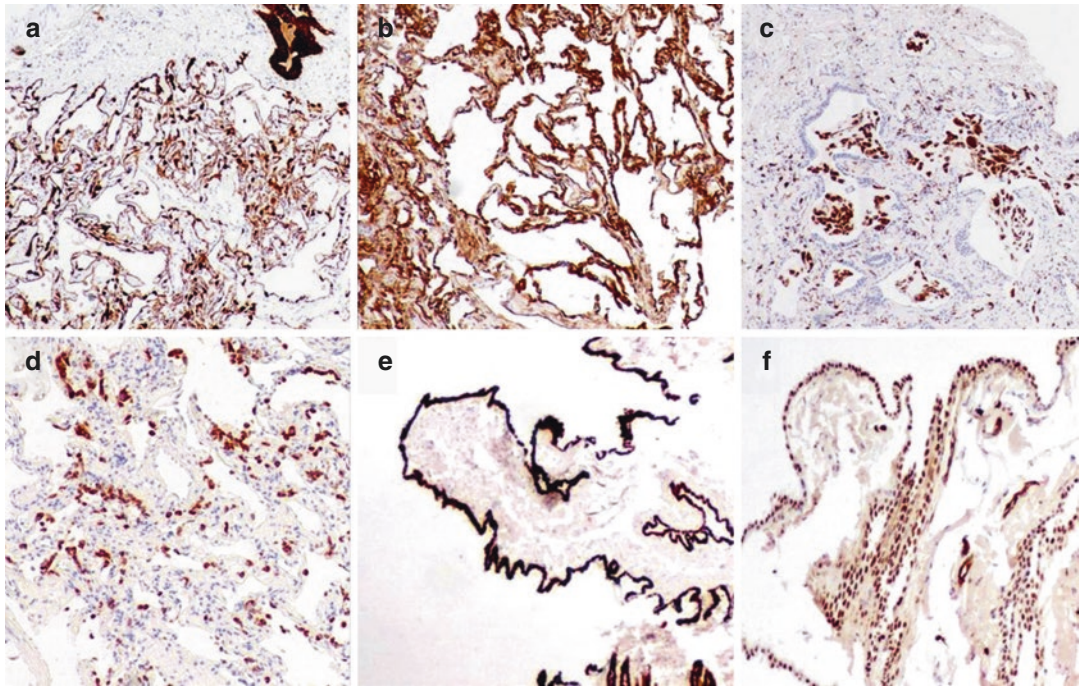

Fig. 9.1 Good quality immunohistochemical stains can be obtained on transbronchial cryobiopsies fixed and paraffin embedded following standard histology protocols: (**a**) cytokeratin 8/18 in alveolar and bronchiolar epithelia; (**b**) CD34 in interstitial vessels; (**c**) CD68 in alveolar macrophages; (**d**) ABCA3 in type II pneumocytes; (**e**) calretinin in mesothelial cells; (**f**) WT1 in mesothelial cells

9.2.1 Immunohistochemical Markers in the Diagnosis of Lung Carcinomas

9.2.1.1 Markers of Pulmonary Epithelial Differentiation (Fig. 9.2)

Cytokeratins (CK) are a large family of proteins, mainly present in intermediate filaments, that are expressed in all epithelial tissues [33]. The biochemical properties of cytokeratins allow their subdivision into two major groups: acidic (n. CK9–CK28) and basic (CK1–CK8) [33]. Within the lung, pneumocytes mainly express low-molecular-weight (LMW) cytokeratins (e.g., CK7, CK8, CK18), whereas airway basal cells express high-molecular-weight (HMW) cytokeratins (e.g., CK5, CK6). Interestingly, during their differentiation into goblet cells and ciliated columnar cells, the airway epithelial precursors loose the HMW cytokeratins that can be re-expressed in "squamous" metaplasia.

Pneumocytes type II (AECII that represent precursors of alveolar epithelium) express a range of proteins that can be successfully demonstrated by immunohistochemistry, including the transcription factor TTF1, the serine protease Napsin A [31, 34], surfactant proteins (especially SP-A), DC-LAMP/CD208, and others [35–38].

TTF-1 belongs to the family of mammalian *NKx2* homeobox genes and encodes for a nuclear transcription factor required for normal development of the thyroid gland and lung epithelial cells, regulating early morphogenesis and branching, as well as inducing later surfactant protein expression by type II pneumocytes. In alveolar pneumocytes, TTF1 is expressed at higher levels when compared to bronchiolar cells, paralleling the expression of several products of TTF1-responsive genes including surfactant proteins A, B, C, ABCA3, and DC-LAMP/CD208 [35–38].

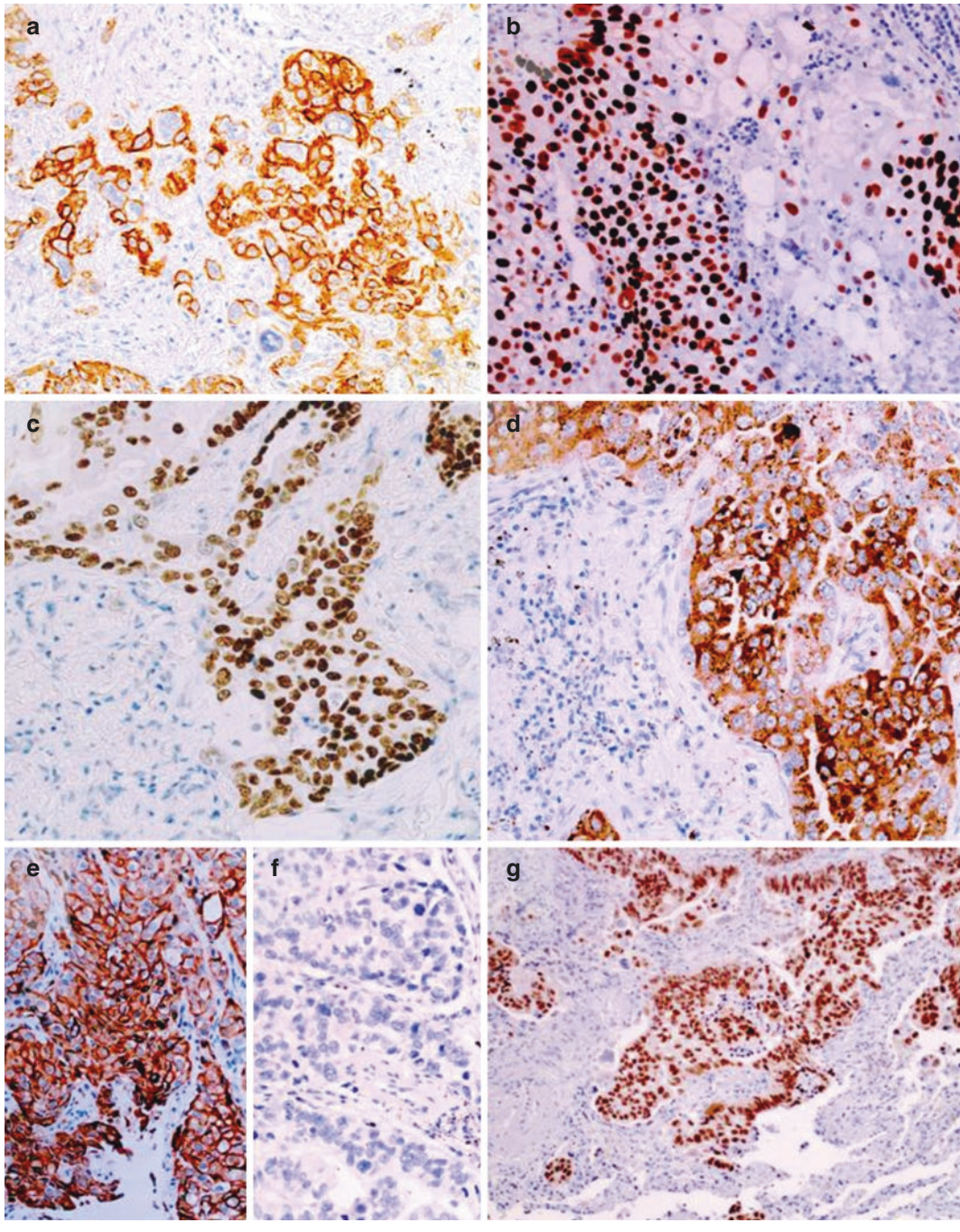

Fig. 9.2 Immunohistochemical characterization of lung carcinomas: (**a**) high-molecular-weight CK5/6 expression in a poorly differentiated squamous cell lung carcinoma; (**b**) p53 overexpression in the same case; (**c**) TTF1 expression in a case of lung adenocarcinoma; (**d**) Napsin A expression in the same case; (**e**) CK7 expression in a case of TTF1-negative (**f**), CDX2 positive (**g**), adenocarcinoma with enteric differentiation

All these markers have been described as useful to positively characterize lung epithelial tumors [34–40]. Lack of expression of these proteins in a proportion of lung carcinomas has been generally considered as a negative feature which decreases markers' sensitivity or specificity. Nevertheless, these molecules can be of value as differentiation markers, since invasive mucinous adenocarcinomas mostly lack their expression, but express the goblet cell-related mucin MUC5AC, and sometimes markers of enteric differentiation (MUC2, CK20, CDX2) [41–45]. This observation can be considered as evidence of a divergent histogenetic derivation (bronchiolar/goblet versus alveolar). The availability of a growing array of "pulmonary," "alveolar," and "bronchiolar" markers can highly improve our understanding of tumor diversity and can be used to better diagnose and classify lung adenocarcinomas [46]. In addition, the decrease or loss of pneumocyte markers is related to the grade of differentiation of conventional adenocarcinomas and can have diagnostic and prognostic value [45, 47].

9.2.1.2 MUC5AC a Marker of Airway Goblet Cells

Goblet cells are increased in the airways as consequence of chronic stimulation (e.g., in smokers). MUC5AC is a secretory mucin typically expressed in bronchial and bronchiolar goblet cells. This mucin is expressed in invasive mucinous adenocarcinoma and is an optimal marker to distinguish this tumor from non-mucinous histotypes [46, 48]. The expression of this mucin in an adenocarcinoma can be considered as an evidence of its "bronchiolar" derivation. MUC5AC is also expressed in adenocarcinomas showing enteric/intestinal differentiation. Interestingly, this entity is highly overrepresented in patients with IPF and carcinoma, and this finding can be considered as evidence of a derivation of these tumors from bronchiolar honeycomb lesions [49].

9.2.1.3 p63 Truncated Isoforms: A Marker of Squamous Metaplasia

The p63 gene is a member of the p53 tumor suppressor gene family playing an important role in the physiological maintenance of different specialized epithelia [50, 51]. Its gene functions are heterogeneous and complex since it undergoes splicing by alternative transcription from two different promoters, producing as many as six distinct isoforms which exert potentially contrasting effects on the same molecular and cellular targets [51]. Transactivating isoforms (the TA-p63 class) maintain a sequence corresponding to the transactivating domain of p53 and have in fact functions similar to p53 in inducing cell cycle arrest and apoptosis. The second class, on the other hand, includes forms lacking the NH_2-terminal domain (ΔN-p63), produced when the p63 gene is transcribed from the cryptic promoter in intron 3. ΔN-p63 forms act as dominant-negative agents toward transactivation by p53 and p63 itself, inhibiting the activity of p53 [52]. When overexpressed, these molecular p63 variants can thus behave as oncogenic molecules. Accordingly, p63 gene amplification and overexpression of ΔN-p63 have been demonstrated in primary lung squamous cell carcinomas [53]. ΔN-p63 (also known as p40) is considered one of the best markers for distinguishing adenocarcinomas from squamous cell carcinomas, together with high-molecular-weight cytokeratins (CK5/6) [10, 54, 55]. ΔN-p63 is also expressed in all cases of thymoma, but not in mesotheliomas [56]. The use of antibodies recognizing all isoforms of p63 (e.g., 4A4) should be used cautiously since the TA-p63 isoform is also expressed by mediastinal B-cell lymphomas [57].

Other IHC tests that should be available for lung cancer diagnosis and characterization include markers of "endocrine" differentiation and proliferation (CD56, chromogranin, synaptophysin, Ki67). These markers are useful for the diagnosis of neuroendocrine lung tumors and also for rare conditions such as endocrine cell hyperplasia [58, 59].

9.2.1.4 Predictive Markers

Intense investigation is currently devoted to find molecular targets for updated therapies in lung carcinoma that need a predictive evaluation of their efficacy, based on the presence of genetic abnormalities and/or the intensity of specific gene product expression. ALK, ROS1, HER2, and others are

the best known among the predictive tests validated for immunohistochemical analysis [60–65]. FISH (fluorescence in situ hybridization) analyses, detecting gene amplifications, deletions, or translocations can be also applied to better define the molecular abnormalities of the neoplastic clones [60, 66–68]. Immunohistochemical analysis has also recently investigated as a predictive tool to maximize the efficacy of immunotherapies [6, 69].

9.2.2 Pleural Pathology (Fig. 9.3)

The differential diagnosis of mesothelial malignancies has been the object of intense investigation, and the use of immunohistochemistry has become a fundamental tool in clinical practice. All international and national guidelines include a panel of immunohistochemical markers that can allow consistent distinction between mesotheliomas and metastatic tumors (mainly lung carcinomas) in order to validate the diagnosis for both clinical and legal issues [70–73].

Immunohistochemical markers can be roughly divided into (1) those confirming the mesothelial origin of a pleural malignancy (e.g., calretinin, WT1, podoplanin/D2–40, and others) [74–76], (2) molecules expressed by pulmonary epithelial carcinomas (BER-EP4, TTF1, CEA, CD15, and others) [77–79], and (3) markers specific for

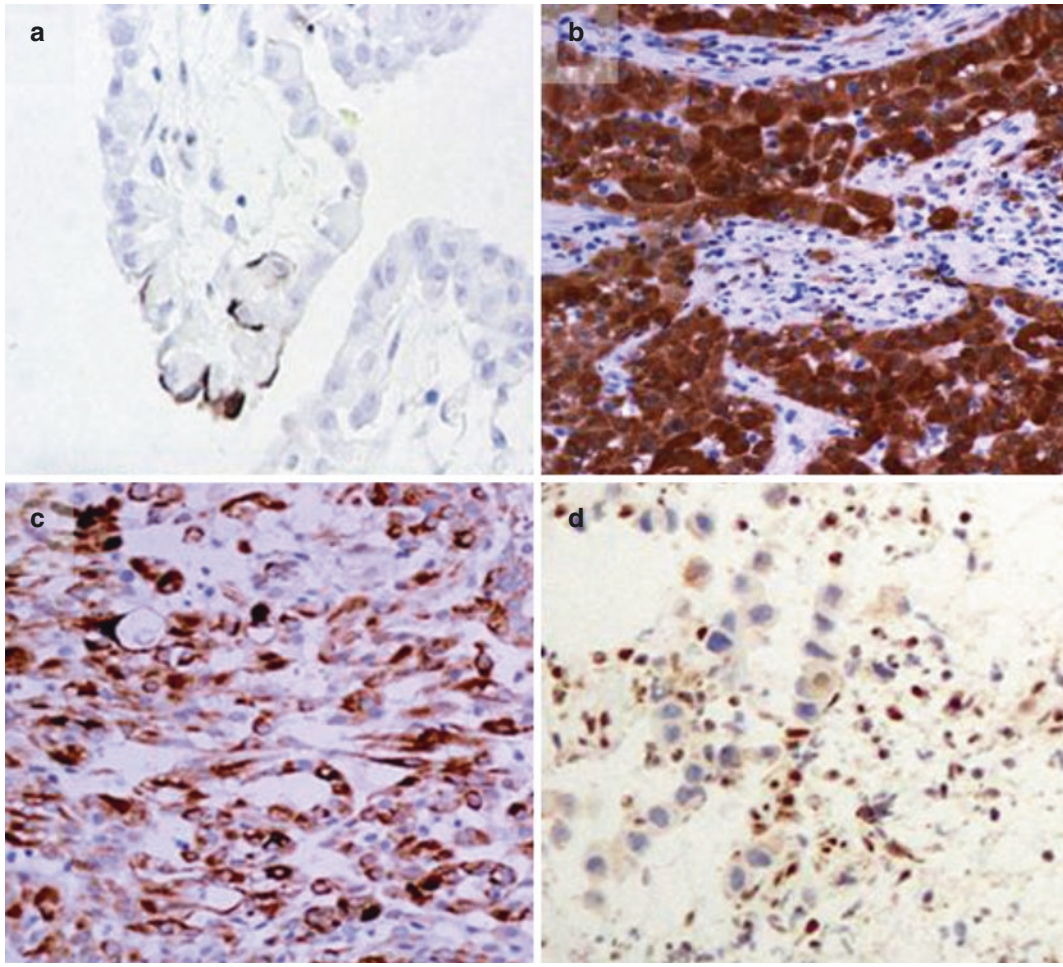

Fig. 9.3 Immunohistochemical characterization of mesothelioma: (**a**) BER-EP4 negative; (**b**) calretinin positive; (**c**) podoplanin positive; (**d**) BAP1 negative (positive internal control is evident)

extrapulmonary metastases of carcinomas (e.g., GATA3, PAX8, CDX2, and others) [32].

In some cases, the distinction between malignant mesothelioma and reactive mesothelial proliferation can be problematic. Advances on the pathogenic mechanisms occurring in mesothelioma development have provided reliable tests for this crucial differential diagnosis, such as the detection of abnormalities affecting p16 and BAP1 genes [73, 80–82]. Loss of the BAP1 nuclear immunoreactivity in mesothelial cells can be considered a robust evidence of malignancy [80–82].

9.2.3 Immunohistochemical Markers in the Diagnosis of Pulmonary Lymphomas

Immunohistochemical analysis is strongly recommended for the characterization of lymphoproliferative disorders occurring in the lung. Among the rare pulmonary lymphomas, the most common subtype is represented by low-grade mucosa-associated lymphoid tissue (MALT) lymphoma, whose immune profile includes the demonstration of the B-cell-specific antigen CD20, the lack of markers specific for other B-cell lymphomas (CD5, cyclin-D1, CD23, etc.), and also the loss of TCL1 expression [83, 84]. A plasma cell differentiation is frequent in MALT lymphoma, and the restricted expression of Ig light chains can represent a reliable diagnostic feature. Lymphomatoid granulomatosis (LYG) is an angiocentric and angiodestructive extranodal lymphoproliferative disease. The histologic presentation of LYG is heterogenous, with variable proportions of large and atypical EBV-infected B cells admixed with reactive T lymphocytes. T-/NK-cell lymphomas can rarely occur in the lungs, and the differential diagnosis between nasal-type T/NK lymphomas and LYG can be difficult, since they share many morphological and immunophenotypic features as angioinvasion, expression of markers of EBV infection, necrosis, and a rich T-cell infiltrate exhibiting cytotoxic immunophenotype, (positivity for CD8, TIA-1, granzyme-B) [83]. Cryobiopsy can be successfully utilized for the diagnosis of pulmonary lymphomas, including the rare endovascular large B-cell lymphomas [85–87].

9.3 Immunohistochemistry in Nonneoplastic Pulmonary Pathology

9.3.1 Immunohistochemical Markers in the Diagnosis of Lung Infections (Fig. 9.4)

A limited number of immunohistochemical markers recognizing infectious organisms (mainly viral) can be applied to paraffin-embedded routine histological samples and can have a diagnostic role in pulmonary pathology. The reagents' panel includes antibodies recognizing polyomavirus, adenovirus, various herpes viruses (herpes simplex virus, cytomegalovirus, human herpesvirus 8, and Epstein-Barr virus). *Pneumocystis jirovecii* can be demonstrated either by Grocott stain or specific antibody [88, 89]. High analytical specificity and sensitivity for mycobacterial detection can be obtained by molecular analysis [90].

9.3.2 Immunohistochemical Markers in the Diagnosis of Interstitial Lung Diseases

The pulmonary microenvironment. When morphological details cannot be easily recognized by H&E staining, some immunohistochemical markers can be useful to precisely define the nature of cells, including epithelial, mesenchymal, and inflammatory cells, as well as extracellular matrix proteins. The epithelial component of the lung tissue can be precisely characterized using cytokeratin subsets, allowing a better evaluation of minimal interstitial changes, pneumocyte hyperplasia, and cell damage or loss (Fig. 9.1). Focal foci of squamous metaplasia can be demonstrated either by ΔN-p63 or high-molecular-weight cytokeratins [91–95]. Surprisingly, CK14 is expressed on type II pneumocytes in focal or diffuse alveolar damage, and

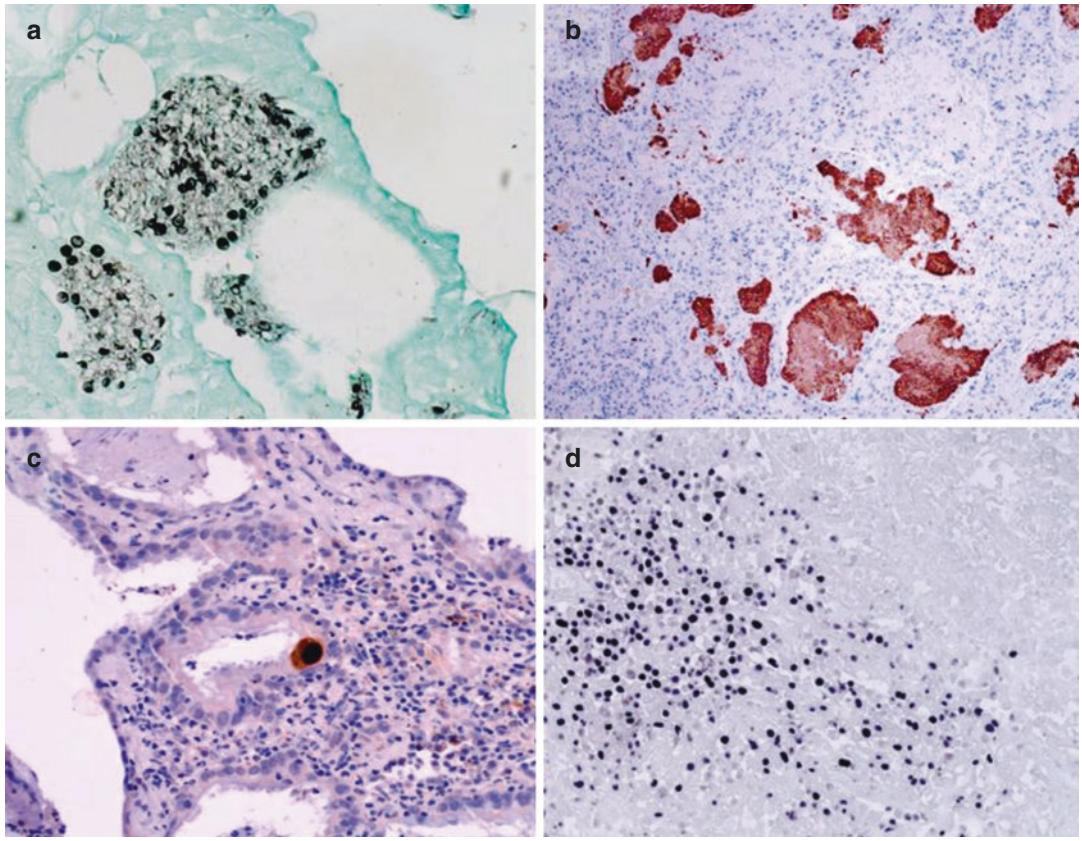

Fig. 9.4 Immunohistochemical characterization of infectious organisms: (**a**) pneumocystis J. (Grocott stain); (**b**) anti-pneumocystis J. immunostaining; (**c**) Cytomegalovirus; (**d**) EBV (EBER in situ hybridization) in a case of pulmonary nasal-type T cell lymphoma

this may be considered a sign of abnormal activation [96]. Different mesenchymal cells can be characterized by markers such as alpha-smooth-muscle actin for smooth muscle and myofibroblasts, tenascin and tubulin beta-3 for myofibroblasts, CD34 for endothelial cells, podoplanin/D2–40 for lymphatic vessels, and others [92, 97, 98].

Increasing information is available regarding the complexity of the immune system. A large number of markers can be applied by immunohistochemical on lung tissue that can precisely detect biologically relevant subsets of lymphoid and inflammatory cells. The classical T-cell lymphoid heterogeneity, based on the expression of membrane antigens such as CD4 and CD8, can now be analyzed in more detail using markers such as GATA3, T-BET, FOXP3, and others, associated to relevant T-cell functional profiles (TH1, TH2, TH17, Treg, etc.), in experimental and clinical studies [99, 100]. Although little evidence has been so far provided on the possible clinical relevance of in situ analyses of these markers, cryobiopsies can provide the samples to better investigate their potential (Fig. 9.5).

9.3.3 Macrophage Markers and Pathology

Macrophages represent a major cell component within the pulmonary microenvironment and exert fundamental roles in maintaining respiratory functions by regulating the surfactant turnover and eliminating infective agents, foreign particles, mucus, dead cell remnants, etc. The majority of macrophages in the normal lung home the alveolar spaces (then they are named alveolar macrophages) and changes of their number, morphology,

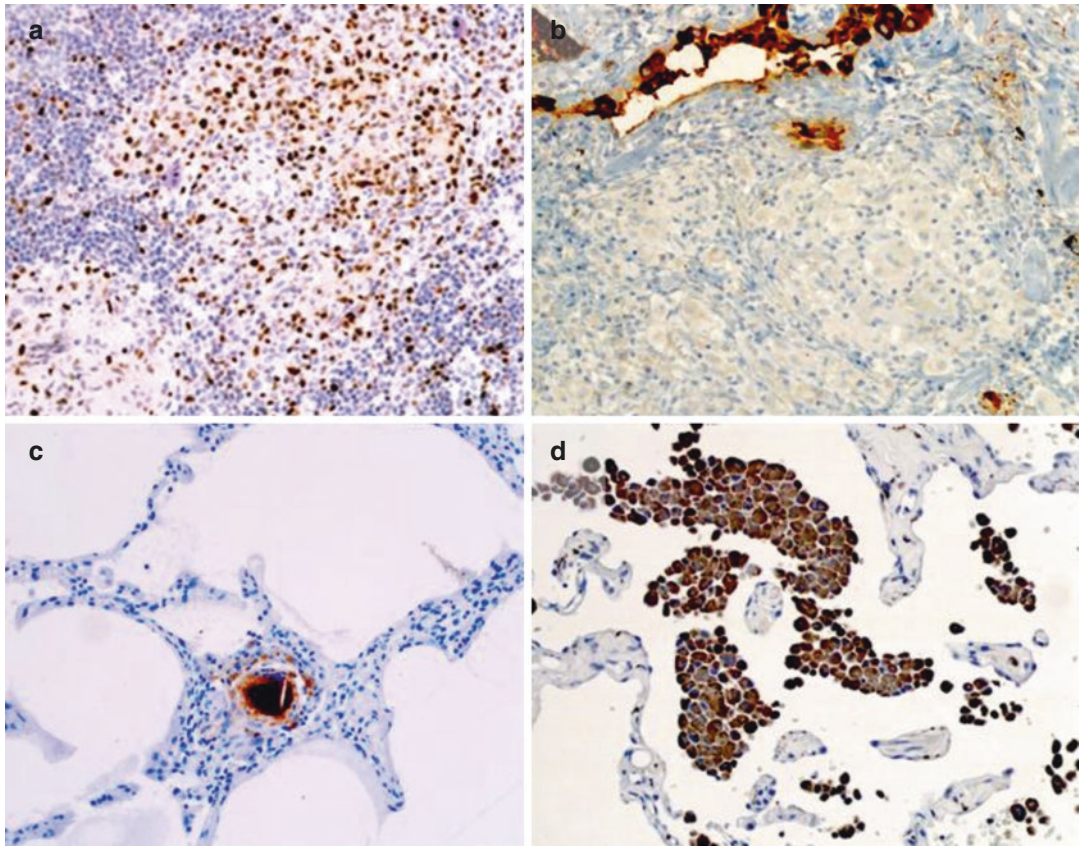

Fig. 9.5 Immunohistochemical characterization of immune-response in situ: (**a**) TH1 T-Bet positive T cells within a sarcoid granuloma; (**b**) sarcoid granuloma macrophages are negative for surfactant-A protein, whereas positive alveolar macrophages show the ingested antigen; (**c**) a small interstitial granuloma evidenced by cathepsin-K immunoreactivity in hypersensitivity pneumonitis; (**d**) clusters of CD68-positive macrophages in Desquamative Interstitial Pneumonia

and/or distribution can provide useful diagnostic information in different pulmonary diseases.

Several markers of alveolar macrophages are available, including *CD68*, *CD11c*, and others. Calgranulins (recognized by the antibody MAC387) are expressed by "young" macrophages, being also expressed by neutrophils [101]. Some antigenic proteins that can be demonstrated by IHC within the cytoplasm of alveolar macrophages are in fact derived by other cell types that are ingested by macrophages, such as the epithelial aspartic peptidase Napsin A and surfactant protein A (Fig. 9.5).

Diseases characterized by abnormalities of alveolar macrophages. Focal or diffuse accumulations of alveolar macrophages characterize smoking-related interstitial lung diseases such as respiratory bronchiolitis-ILD and desquamative interstitial pneumonia. Clusters of foamy macrophages (engulfing surfactant proteins and lipids) can be related to diseases of disturbed surfactant turnover (e.g., diffuse panbronchiolitis or in amiodarone lung disease).

Diseases characterized by accumulation of extrapulmonary macrophages. In some pulmonary lesions, the accumulation of macrophages is due to the recruitment of circulating monocytes that eventually differentiate in the lung interstitial spaces. These include a variety of inflammatory diseases and can be divided in granulomatous and non-granulomatous.

Granulomatous diseases. A particular type of macrophage activation leads to the formation of epithelioid granulomas. This reaction is triggered

by TH1 lymphocytes and characterizes different pulmonary granulomatous diseases including immunological (sarcoidosis, berylliosis, hypersensitivity pneumonitis) and infective diseases (mycobacteriosis, etc.). Epithelioid and giant cells in granulomas are not modified alveolar macrophages but directly derive from circulating monocytes. The phenotype of epithelioid cells is different from that exerted by alveolar macrophages (negative for ZAP 70, Napsin A), and these markers can occasionally be used to help in difficult cases. Epithelioid macrophages are activated cells and express molecules that are up-modulated during activation. *Cathepsin K*, a protease expressed at high levels in bone marrow osteoclasts and in granuloma macrophages, can be useful in detecting small granulomas as in most cases of hypersensitivity pneumonitis [102] (Fig. 9.5).

9.3.3.1 BRAF-Related Histiocytoses

Recently, it has been demonstrated that Langerhans cell histiocytosis (both non-pulmonary and pulmonary—PLCH) and Erdheim-Chester disease are pathogenetically related to a mutation affecting the BRAF oncogene [103, 104]. This finding is relevant since it can be considered as evidence of the "clonal/neoplastic" nature of these histiocytoses (previously considered as "inflammatory") and also provides a robust diagnostic tool, either utilizing immunohistochemistry or molecular analysis [105, 106]. Interestingly, in both these diseases, oncogene-induced cell senescence (OIS) has been suggested as a pathogenic feature, this explaining the SASP (senescence-associated secretory phenotype)-related secondary inflammatory features of the diseases [106, 107]. Demonstration of senescence-related markers such as $p21^{waf1}$ and p16 can provide in this context prognostic information [106]. Pulmonary Langerhans cell histiocytosis is characterized by accumulation of CD1a-positive and CD68-negative cells, whereas Erdheim-Chester histiocytes are typically CD68 positive and CD1a negative.

9.4 Lymphangioleiomyomatosis

Lymphangioleiomyomatosis (LAM) is a rare disease that affects the lungs of women, usually in premenopausal age. The disease is progressive and potentially fatal. The LAM cells, which harbor mutations in tuberous sclerosis genes, progressively infiltrate the perilymphatic spaces of the lung parenchyma. The phenotype of LAM cells is peculiar, since they express alpha-smooth muscle actin and desmin, together with melanocytic markers such as HMB45, HMSA-1, MelanA/Mart1, microphthalmia transcription factor (MITF), and cathepsin k [28, 108]. This distinctive phenotypic profile can be useful to precisely characterize the disease on small transbronchial biopsies [109] (Fig. 9.6).

9.4.1 Immunohistochemistry in the Diagnosis of Idiopathic Pulmonary Fibrosis and DPLD

The diagnosis of idiopathic pulmonary fibrosis (IPF) is classically based on algorithms that include a multidisciplinary evaluation of clinical, radiological, and histological data. When the radiological pattern is definite for usual interstitial pneumonia (UIP), an invasive approach is discouraged in the consensus protocols. The introduction of TCB can help in providing a definite diagnosis of UIP in those cases where the data are dubious or not consistent for UIP, thus avoiding either SLB or renounce to a certain diagnosis. The use of some markers related to the pathogenesis of IPF can help in some difficult cases. To date, there is a wide consensus on the assumption that IPF is not an inflammatory disease, but its pathogenesis is related to an accelerated senescence affecting pneumocytes that progressively reach a status of stem cell insufficiency at particular sites [110–113]. The causes of this intrinsic and irreversible defect are multifactorial, including age-related telomere attrition, a genetic predisposition (that is predominant in familiar cases), together with the chronic exposure to toxic substances (e.g., cigarette smoke), and also an anatomic component related to mechanical stress [114]. Immunohistochemistry can be useful in detecting early evidence of pneumocyte senescence. The expression of cell senescence-related markers such as p16, p21, and beta-galactosidase [111, 115–117] in hyperplastic type II pneumocytes

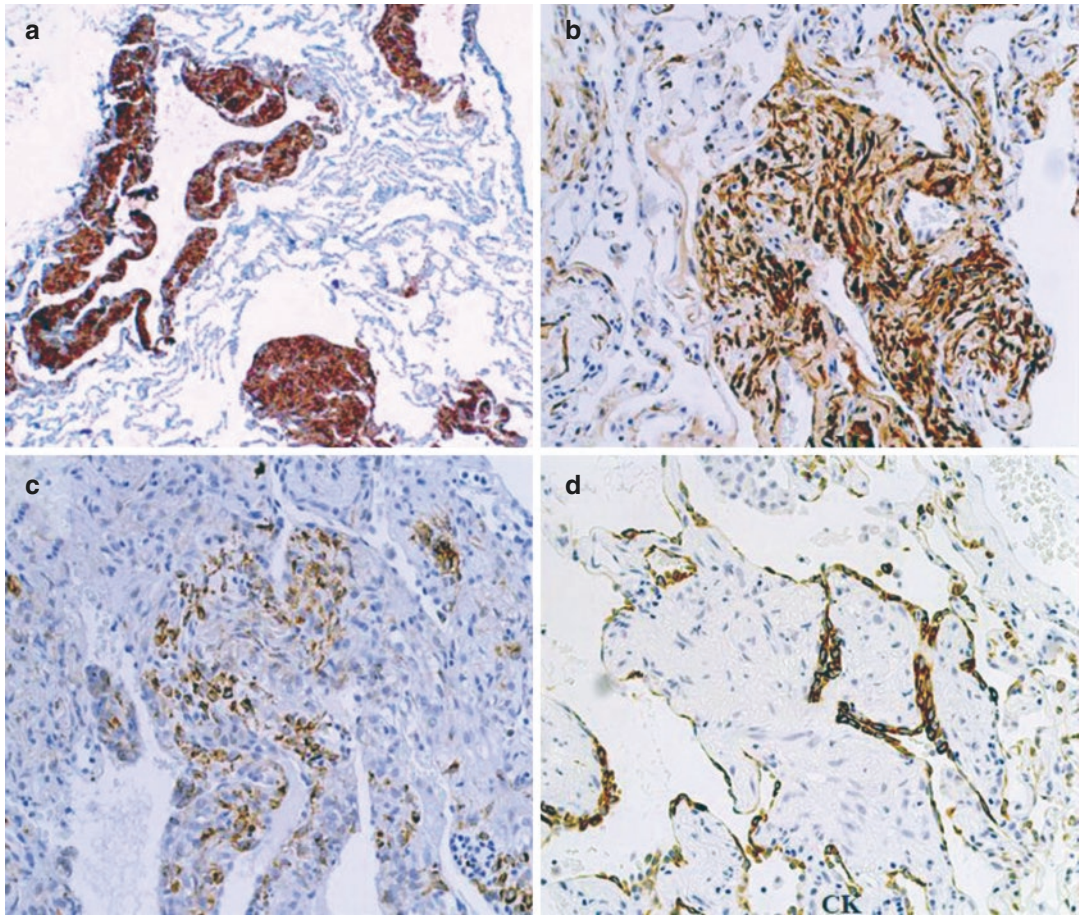

Fig. 9.6 Immunohistochemical characterization of lymphangioleyomiomatosis (LAM) cells: (**a**) cathepsin-K positive; (**b**) alpha-smooth muscle actin positive; (**c**) HMB45 positive; (**d**) cytokeratin 8/18 negative

represents the evidence of focal alveolar damage in IPF and also on small TCB (Fig. 9.7).

The use of other markers can help to better visualize small fibrotic lesions that can be missed on H&E morphology, together with details demonstrating abnormalities, including epithelial-mesenchymal transition (EMT) and abnormal angiogenesis, occurring in microenvironmental organization of the normal parenchyma [118]. Several myofibroblastic markers have been described, including the extracellular matrix protein tenascin, and its immunohistochemical evaluation can have prognostic significance in pulmonary fibrosis [98, 119]. Tubulin beta-3 has been recently proposed as a reliable immunohistochemical marker of myofibroblast foci in IPF [120]. This marker in fact is expressed in both myofibroblasts and epithelial cells exhibiting EMT in fibroblast foci, together with a variety of molecules aberrantly expressed within the fibrotic lung tissue (ZEB1, TWIST, beta-catenin, and others) [118, 120–124]. A useful immunohistochemical finding is observed within honeycomb lesions that we named "sandwich foci" because of the peculiar three-layer structure formed by myofibroblasts, basal cells expressing laminin-5 γ-2 chain, and heat shock protein hsp27 [122] (Fig. 9.7). Although these findings appear useful diagnostic features in the differential diagnosis of IPF and other DPLD, they need to be validated on large case series.

9.4.1.1 Microscopic Honeycomb Lesions

Micro-honeycombing is a major morphological feature of IPF, although it is not requested by consensus statements for its histological diagno-

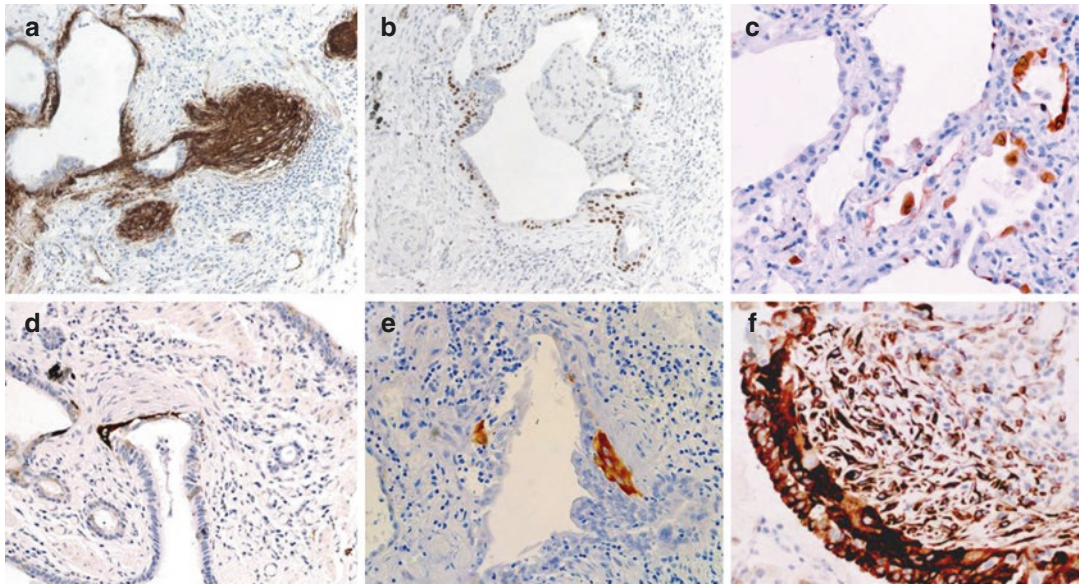

Fig. 9.7 Immunohistochemical features in IPF: (**a**) strong tenascin expression in fibroblast foci; (**b**) abnormal distribution of ΔN-p63 basal cells in bronchiolar honeycombing; (**c**) focal expression of the cell senescence-associated marker p16 in type II pneumocytes; (**d**) laminin-5 γ-2 chain expression in "sandwich" fibroblast foci; (**e**) heat shock protein 27 in sandwich foci; (**f**) strong tubulin beta-3 in both luminal epithelium and fibroblast foci

sis when imaging is consistent with the diagnosis of UIP pattern. In honeycomb lesions the bronchiolar structures that are close to parenchymal fibrotic area are progressively changed showing distortion, enlargement, mucous accumulation, and abnormal proliferation. Fibroblast foci are frequently observed within these lesions, showing a three-layered *sandwich* structure (see above). The mucous accumulation within these lesions always contains the mucin MUC5B, regardless of the occurrence of the MUC5B polymorphism (a well-known predisposing genetic feature of IPF) [125–127]. This finding is typical of IPF micro-honeycombing. In addition, micro-honeycomb lesions in IPF show several abnormalities that can be demonstrated by immunohistochemistry, including increased expression of WNT/beta-catenin targets such as MMP7, cyclin-D1, and MYC, as well increased expression of senescence-related markers p16 and p21 [111, 117, 121]. These abnormal features are likely related to the development of epithelial malignancies with bronchiolar phenotypes in IPF [49].

9.4.1.2 Molecular Analysis in IPF

The demonstration of an increasing number of gene abnormalities (affecting genes involved in surfactant synthesis, telomerase functions, MUC5B polymorphisms, telomere length, etc.) in familiar and sporadic IPF is crucial for the understanding of the pathogenesis of these devastating diseases and may provide a new perspective for their classification, diagnosis, and prognostication [128–131].

9.5 Diffuse Alveolar Damage (DAD)

In diffuse alveolar damage (DAD), the morphological pattern characterizing acute respiratory distress syndrome, the pneumocyte type II hyperplasia is generalized and diffuse, and markers of EMT are easily demonstrated, including nuclear beta-catenin, slug, tubulin beta-3, CK14, and others (Fig. 9.8) [96, 120–123].

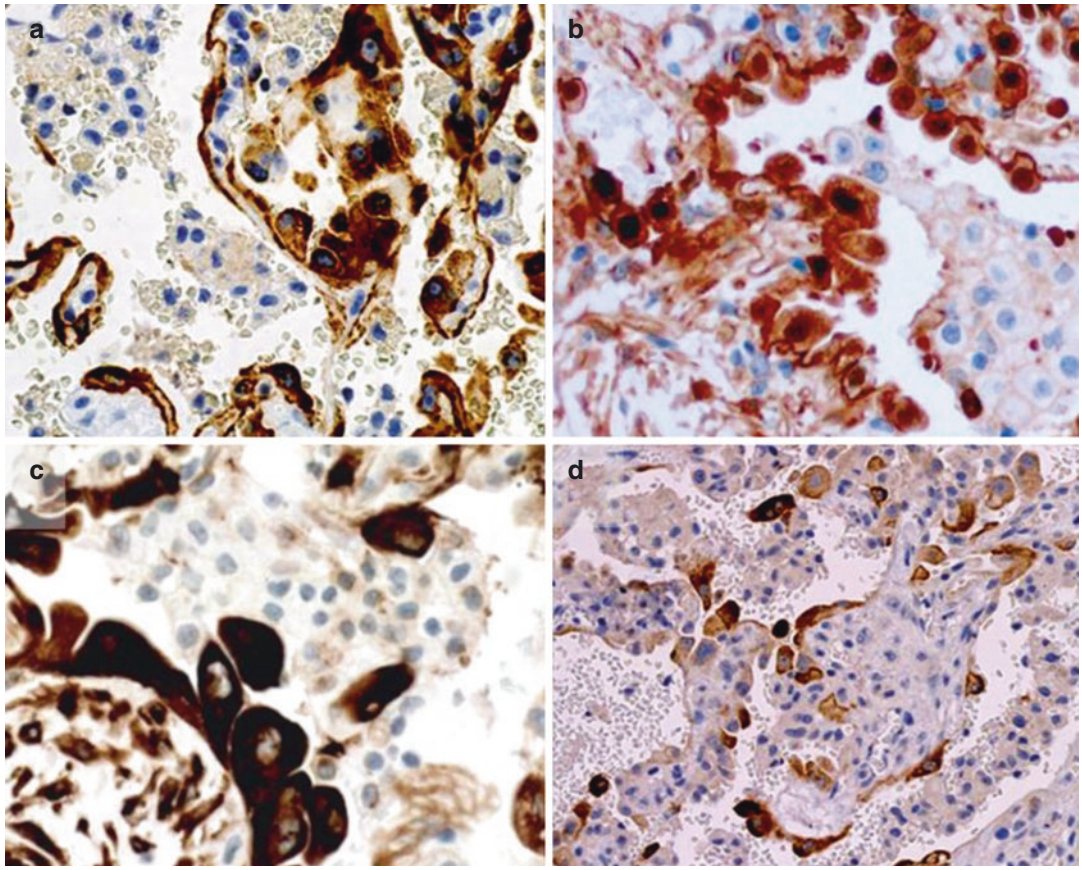

Fig. 9.8 Immunohistochemical characterization of hyperplastic type II pneumocytes in diffuse alveolar damage: (**a**) cytokeratin 8/18; (**b**) nuclear and cytoplasmic accumulation of beta-catenin; (**c**) strong immunoreactivity for tubulin beta-3 in both epithelial cells and myofibroblasts; (**d**) laminin-5 γ-2 chain expression in activated pneumocytes

9.6 Pleuroparenchymal Fibroelastosis (PPFE)

PPFE is a severe interstitial disease characterized by progressive effacement of the pulmonary parenchyma with pleural/subpleural fibroelastosis often accompanied by interstitial thickening and remodeling. Although PPFE has been included within the idiopathic interstitial pneumonias [132], its identity is still matter of debate [133, 134]. Relevant to diagnosis is the demonstration of abnormal interstitial accumulation of elastic fibers that can be evidenced either using elastic Van Gieson's stain or, more precisely, with elastin-specific monoclonal antibodies. Recently, the occurrence of podoplanin-reactive myofibroblasts has been proposed as a specific immunohistochemical staining to distinguish between PPFE and IPF abnormal remodeling of the lung parenchyma [135].

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