Molecular diagnosis in lung diseases

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1. ABSTRACT

The development of different molecular biology techniques in the past decade has led to an explosion of new research in molecular pathology with consequent important applications to diagnosis, prognosis, and therapeutics, as well as a clearer concept of the disease pathogenesis. Many methods used in molecular pathology are now validated and used in several areas of pathological diagnosis, particularly on infectious and neoplastic diseases. The spectrum of infectious diseases, especially lung infective diseases, is now broadening and modifying, thus the pathologist is increasingly involved in the diagnosis of these pathologies. The precise tissue characterization of lung infections has an important impact on specific therapeutic treatment. Increased knowledge of significant alterations in lung cancer has led today to a better understanding of the pathogenic substrate underlying the development, progression and metastasis of neoplastic processes. Molecular tests are now routinely performed in different lung tumors allowing a more precise patient stratification in terms of prognosis and therapy. This review focuses on molecular pathology of the principal infective lung diseases and tumors.

2. INTRODUCTION

Molecular pathology, defined broadly as the use of nucleic acid probes to diagnose and study disease, is an emerging discipline of growing importance and promise (1). Molecular techniques from more simple to high throughput are becoming fundamental ancillary tools to improve final morphological diagnoses. Traditionally, snap-frozen samples are considered optimal for nucleic acid extractions. In the past, the capacity to extract high quality DNA and RNA from formalin-fixed paraffin-embedded (FFPE) tissue specimens was considered cumbersome and an important limiting factor for routine use of molecular methods (2,3). To overcome this problem, in the last few years a multitude of reliable protocols for DNA, RNA and protein extraction from FFPE tissue have been developed and the use of neutral buffered formalin for 24–48 hours can be regarded as a standard procedure for molecular approaches with high sensitivity and specificity. The current use of molecular tools across the entire spectrum of human diseases provides a more precise diagnostic characterization and overall important suggestions for better patient stratification and treatment.
The present review will briefly mention the most common molecular approaches used in routine pathological practice, and then will focus on the main infective and neoplastic lesions whose final diagnosis is currently associated with molecular analyses. Both the sections (infective and neoplastic lesions) will be preceded by a brief introduction reporting general aspects of molecular pathology and the main infective lung diseases and tumors.

3. MOLECULAR TECHNIQUES

3.1. In situ hybridization (ISH)

In situ techniques, particularly ISH, are techniques that allow precise localization of a specific segment of nucleic acid within a histological section. The underlying basis of ISH is that nucleic acids, if adequately preserved within a histological specimen, can be detected through the application of a complementary strand of nucleic acid to which a reporter molecule is attached. Visualization of the reporter molecule allows DNA or RNA sequences to be localized in a large variety of biological samples, both tissue and cytological specimens. This approach is particularly used when it is crucial to describe the exact cellular localization of DNA or RNA of interest (4). There are several limitations to the use of this approach, in particular the low sensitivity and reliability. ISH is cumbersome: specimen preparation consists of securing the sample to glass slides and denaturing the DNA without detaching or destroying the morphological identity of the cells. Moreover, when using fluorescence ISH, the fluorescent signal rapidly fades over time, a fluorescence microscope is needed for interpretation (which is not usually done in routine clinical works) and it is difficult to detect the overall morphology and tumor heterogeneity.

3.2. Polymerase chain reaction (PCR), reverse-transcriptase (RT)-PCR

Non-in situ techniques are emerging as the preferred approach for molecular diagnosis of both oncological and infectious diseases. PCR is a primer-mediated enzymatic amplification of specifically cloned or genomic DNA sequences (5). Different strategies have been introduced to perform this analysis also in archival tissues (6). Throughout the last decade numerous works have demonstrated that it is also possible to sensitively detect mRNA levels (i.e. profile gene expression) using FFPE as a source of RNA, despite the fact that RNA extracted from archival tissue is often present in fragments less than 300 bases in length (7,8).

The availability of adequate quality and quantity of RNA extracted and reverse-transcribed (by RT-PCR) in cDNA, also from FFPE tissues, is extremely important. The prognostic/predictive potential of gene expression profile, i.e. defining new subcategories of known diseases with different prognoses and possible dissimilar responses to drugs, is now evident and reported by several groups (9,10). PCR and RT/PCR, surely the most frequent diagnostic tools used in molecular labs, are characterized by high sensitivity and reliability but present some disadvantages such as possible contamination, since it leads to an exponential increase of the gene target copy number, and the laborious post-amplification processing (e.g. gel electrophoresis). Over the years several creative pre- and post-amplification methods have been developed to prevent or reduce amplicon carryover contamination.

3.3. Real-time PCR

A significant advance in PCR was the invention of real-time PCR, which allows for multiple analysis detection and quantification in a single tube (12). One approach for real-time monitoring of amplicon production is to use fluorescent DNA-intercalating dyes, such as SYBR Green I, which binds non-specifically to double-stranded DNA generated during amplification. A more popular alternative approach is to use a fluorescent-labeled internal DNA probe which specifically anneals within the target amplification region (13). Moreover it eliminates the need for laborious post-amplification processing (e.g. gel electrophoresis) conventionally needed for amplicon detection (14). Real-time PCR offers a detection method that is rapid, specific, and sensitive. In the past number of years, an abundance of multiplex real-time PCR and reverse transcriptase (RT) real-time PCR products have become available on the clinical diagnostic market (15). However, some limitations have been detected in this technique, such as the overlap of emission spectra and the increased risk of false-negative results (15). Different commercial kits both for infective agent detection (i.e. artus EBV TM PCR Kit, Qiagen) and for EGFR and KRAS mutation analysis (cobas® EGFR Mutation Test, Roche Molecular Systems Inc., therascreen® EGFR RGQ PCR Kit, Qiagen, therascreen® KRAS RGQ PCR Kit, Qiagen) are now available and are usually CE-IVD guaranteed, indeed real-time PCR shows a much higher successful detection rate and mutation rate of lung cancer tissues compared with that of Sanger sequencing. The spectrum of tested
agents and the technology used to reveal the PCR products as well as the laboratory organization are determinant for the selection of the most appropriate kit (16).

3.4. Gene sequencing

DNA sequencing has revolutionized biological and medical research and has now become an essential tool of diagnostic molecular pathology. Gene sequencing consists of an enzymatic reaction that polymerizes the DNA fragments complementary to the template DNA of interest (unknown DNA). DNA polymerase catalyzes polymerization of deoxynucleoside triphosphates (dNTP) onto the DNA until the enzyme incorporates a modified nucleoside into the growing chain. Automated DNA sequencing utilizes a fluorescent dye to label the nucleotides instead of a radioactive isotope, thus reducing the volume of low-level radioactive waste and providing more reliable research results than manual DNA sequencing (17). Sanger sequencing was long considered to be the “gold standard” method for mutational analyses. However this method has several limitations: low throughput (in the oncological field, requires at least 30%–40% of neoplastic to non-neoplastic cell ratios) and high cost making multigene panels laborious and expensive. A number of alternative technologies based on real-time PCR (PCR, allele-specific, melting curve, PNA clamp) strip assay, chip array, pyrosequencing, and next-generation sequencing have been developed to increase the sensitivity of mutational analyses, allowing investigation of poorly enriched tumor samples below the detection threshold for Sanger sequencing (18).

“Next-generation” and “massive-parallel” DNA sequencing are blanket terms used to refer collectively to the high throughput DNA sequencing technologies available which are capable of sequencing large numbers of different DNA sequences in a single reaction (19). Since this approach improves the detection of mutations, it should be promoted for the clinical diagnosis of mutations in specimens with unfavorable tumor cell content (20), e.g. next generation sequencing platforms by Life Technologies, Illumina-Solexa, Diatech, Roche and many others. These analyses should be performed by very experienced molecular lab. In these setting robust mechanisms of external quality assessment to the molecular procedures and standardized protocols should not be underestimated.

Comparative genomic hybridization is a molecular cytogenetic method for comparing of two genomic DNA samples arising from two sources, which are most often closely related, because it is suspected that they contain differences in terms of either gains or losses of either whole chromosomes or subchromosomal regions. Conventional and array CGH has been used mainly for the identification of genetic abnormalities that are recurrently lost or gained in tumors; in the future, their use could be extended for diagnostic, prognostic and therapeutic purposes.

High-throughput sequencing approaches are also a method of choice for a large spectrum of known and unknown viruses discovery but it will also become a tool for in depth or even routine diagnosis as soon as the cost and delays decrease significantly (9).

4. MOLECULAR PATHOLOGY OF INFECTIVE LUNG DISEASES

Infectious diseases account for the majority of human diseases and are still the leading cause of debilitating chronic disease and death. In the last few decades, medicine has made giant steps in the diagnosis and treatment of infection but it has also seen the emergence of new and deadly pathogens that continue to have a significant impact on modern medicine. New medical treatments such as potent immunosuppressive treatment that weaken host defenses have contributed to the emergence of new microorganisms and overall to the recrudescence of others. In this scenario the pathologist is increasingly needed to provide diagnoses from both cytology and biopsy specimens. Traditional microbiological investigations such as culture have several important limitations: they are not able to distinguish infection from colonization, nor can they determine the significance of isolated microorganisms. Only the presence of a pathogen in situ together with an inflammatory response by the host represents acceptable evidence of its role in infection. A high level of experience and expertise in this field together with the use of different ancillary tools, as molecular investigations, are mandatory for a sensitive final diagnosis. The use of molecular tools in routine pathology significantly increases the sensitivity of the diagnosis mainly for two reasons: 1) exiguous specimens are frequently available for pathological diagnosis due to limited applicability of invasive procedures in debilitated/immunocompromised patients, 2) a small number of pathogens, frequently
also modified in their morphology by antimicrobial therapy are present in specimens. Exact etiological characterization is extremely important for target therapy and molecular monitoring of infective agents is now adopted to verify the efficiency of treatment. Infective pneumonia is a frequent cause of morbidity and mortality in children, immunocompromised patients and in the elderly. Respiratory tract infections remain difficult to diagnose accurately as a broad range of pathogens and opportunistic microorganisms are involved in their etiology. Frequently, patients with signs of respiratory tract infections are treated empirically without an accurate diagnosis of the causative microorganism. Delays in accurate pathogen-specific diagnoses may result in the prescription of inappropriate medical treatment and poor outcome. The pathologist has an important role in the diagnosis or exclusion of infectious diseases mainly for those developing in immunocompromised patients such as lung recipients. Indeed lung allografts show increased susceptibility to bacterial and opportunistic infections, especially when compared with recipients of other solid organ transplants (21). Post-transplant lungs are continuously exposed to the environment, have impaired mucociliary clearance and a decreased cough mechanism because of denervation of the allograft, and are treated with high-dose immunosuppression, all of which increase the risk for infection (22). Numerous bacterial, viral, and fungal pathogens take advantage of these factors to cause invasive disease. Although some microorganisms or their cytopathic effects may be clearly visible on routine hematoxylin and eosin-stained sections, additional histochemical or immunohistochemical stains and overall molecular investigations are often needed for a definitive characterization.

Virus, mycobacteria and fungi, the most frequent pathogens investigated in lung specimens by using also molecular tools, will be more thoroughly discussed below.

4.1. Viral infection

Viral infections, including beta-herpes viruses, particularly cytomegalovirus (CMV) and community respiratory viruses-CARVD- (rhinovirus, influenza virus A/B, parainfluenza virus 1/2/3, respiratory syncitial virus) are frequent pathogens in immunocompromised patients, particularly in HIV-seropositive and lung transplant recipients. Notably in the lung transplant field the recent development of molecular methods for CMV detection has resulted in more rapid diagnosis and has played an important role in the management of CMV infection. Quantitative measurements of viral load in blood can trigger initiation of antiviral therapy or can be used to monitor response to therapy (23,24). While several works have emphasized the importance of molecular diagnostic tests on blood (25) it was also demonstrated that blood viral load appears useful but not completely predictive of CMV pneumonitis (26). Transbronchial biopsies and bronchoalveolar lavage specimens remain the best tools for the final diagnosis of infective viral pneumonitis. While bacterial, fungal or protozoal microorganisms are more easily detectable, viral infections are quite hard to characterize by routine histological and cytological examination. In post-transplant transbronchial biopsies it is important to note that cytological or histological interpretation appears fairly difficult due to three main aspects: precise identification of viral infections with histological patterns that may differ from those observed in the non-transplanted immune-competent patient, differentiation of acute rejection from viral infections and differentiation between colonization, subclinical infection and clinically significant disease. Viral cytopathic effects, including the typical cytomegalic changes, are rarely seen in post-transplant biopsies or cytological smears.

The use of immunohistochemistry, with both monoclonal and polyclonal antibodies, has significantly increased the sensitivity for the histological diagnosis of CMV pneumonitis compared to standard hematoxylin and eosin staining. ISH has been demonstrated to have similar sensitivity as immunohistochemistry (27,28) and this is also our recent experience (Calabrese et al., unpublished data). All our cases tested with CMV immunohistochemistry showed the same marked cells in the serial sections processed by CMV ISH. Thus ISH due to greater technical difficulties and higher cost is rarely used in routine CMV screening. Routine histology with immunohistochemistry and/or ISH show however low sensitivity in CMV detection often leading to under-diagnosis and/or delayed diagnosis of CMV pneumonitis (29). Increasing interest has focused on more sensitive molecular detection techniques both applied to transbronchial biopsy and overall to bronchoalveolar lavage.

Although the critical threshold of viral load (4.6. x 10^4 and 5.0. x 10^5) is not yet completely validated (30), several works have proposed CMV quantification in bronchoalveolar lavage to have a higher sensitivity in the differentiation of
patients with active CMV disease from latently infected patients (31) and suggested that molecular qualitative assay on bronchoalveolar lavage should replace traditional culture and also CMV DNA assay in the blood. Regarding this aspect several authors have demonstrated a significantly higher sensitivity of CMV DNA in bronchoalveolar lavage compared with the same detection in plasma samples. High bronchoalveolar lavage CMV load (>64,000 copies/ML) has been reported to be associated not only with viral inclusions in lung allograft biopsies but also is more likely to be associated with specific symptomatic disease syndromes such as CMV pneumonitis (32). However, a low bronchoalveolar lavage cellularity (due to the technical procedure) could represent a bias in the interpretation of real-time PCR quantification results. Molecular tools, in particular the most sensitive PCR or quantitative PCR, have recently been reported to have an important impact also on the diagnosis of CARV, today recognized as an important cause of morbidity and sometimes mortality in immunocompromised patients. In healthy subjects these infections are generally limited to the upper respiratory tract with viral clearance occurring within a few days (33). In contrast, patients with impaired immune function are at higher risk of severe lower respiratory illness with prolonged shedding (34). Among CARV the most frequently detected virus is rhinovirus. Rhinovirus is mostly limited to the upper respiratory tract but experimental data have shown that it also replicates in the lower respiratory tract (35) and severe rhinoviral pneumonia has been described in immunocompromised hosts (36), including lung transplant recipients. Moreover molecular investigations using the RT-PCR and gene sequencing portion of the 5’NCR region and VP1 surface glycoprotein were also able to demonstrate rhinoviral persistence in lung transplant recipients with lung dysfunction (37) suggesting a possible influence of this pathogen on chronic allograft dysfunction (see below, section “contributing etiological role”).

4.2. Mycobacterial infection

Mycobacterial infections have a high economic human and animal health impact. The incidence of nontuberculous and tuberculous mycobacterial diseases has increased worldwide over the past several decades. Symptomatic and severe disease commonly occurs in patients with structural lung diseases, such as chronic obstructive pulmonary disease, bronchiectasis and immunodepressed patients. Early diagnosis of mycobacterial infection, particularly tuberculosis (TB), is extremely important for starting prompt and specific antimycobacterial treatment. In terms of histopathological diagnosis, TB can be diagnosed only as “a chronic granulomatous inflammation, suggestive of tuberculosis” on a routine surgical pathology report. However, histopathological features of chronic granulomatous inflammation can be detected in different conditions and diseases other than TB, such as foreign body reaction, fungal infection, sarcoidosis, and Wegener’s granulomatosis (38). Although additional morphological findings can help to perform a more accurate diagnosis (i.e., presence of vegetable material for foreign body reaction, or necrotizing vasculitis in Wegener’s granulomatosis), the tissue reaction to mycobacteria, including TB, shows a spectrum of changes that may not involve the presence of pathognomonic granuloma. Chronic inflammation without distinct granulomatous lesions may be the only histological feature detected in several cases of mycobacterial infections including TB forms. In addition, since the size of histological specimens is usually small as bronchial or tranbronchial biopsy, all pathognomonic histopathological features of TB, such as a well-formed granuloma with caseous necrosis, are rarely seen. The conventional methodology of microscopic examination for acid-fast bacilli, culture, biochemical tests is laborious and time-consuming. In particular routine microscopic evaluation of lung tissue stained with both types of acid fast stains (Ziehl-Neelsen and rhodamine-auramine stains) shows a low sensitivity also when the lesion appears histologically active or many bacilli are detected in the sputum. Some organic solvents, which are used to make paraffin-embedded tissue samples, have been suggested to affect the stainability of mycobacteria by acid-fast staining. This hypothesis seems reasonable because the molecular target of acid-fast staining dyes (fuchsin, auramine, or rhodamine) is the mycolate on the bacterial surface (39). Mycolate is soluble in organic agents (40) and might be more or less extracted from the cell surface into the liquid. Similarly, immunohistochemical identification using polyclonal antibodies against mycobacteria proved to be not sensitive enough for this purpose (41). Moreover acid fast staining and immunohistochemistry are not able for distinguishing MT from non-tuberculous mycobacteria (NTM).

Over the last few years, new molecular methods have been introduced, including nested-PCR, RT-PCR, real-time PCR, DNA sequencing.
and DNA strip technology, leading to considerable improvement in both the speed and accuracy of mycobacterial identification (42). All these could be used also in FFPE applying simple protocols that take into consideration DNA fragmentation and/or cross-linkage caused by formalin fixation (43). In addition to FFPE-related problems mycobacteria carry a further methodological obstacle because of their thick mycolate-rich outer cell wall, which requires special measures of cell disintegration without causing further damage to DNA (44). The highest sensitivity for MT and NTM detection is usually obtained amplifying IS6110 and HSP65 genes respectively (45,46). The study of Fukunaga et al. reported a significant low sensitivity of acid fast staining compared to real-time PCR. The estimated number of bacilli from the real-time PCR data was $10^3$ to $10^9$ times larger than the number counted with microscopy among the acid fast staining positive samples (47). In our experience less than half of TB-PCR–positive cases have the typical lesion of granulomatous inflammation with central necrosis. For this reason, if there is a high suspicion of mycobacterial infection based on both clinical and radiological findings, in our center we routinely perform nested-PCR method, especially in cases with small biopsy specimens or cases of immunosuppressed patients, often lacking of typical granulomatous lesions. Nested PCR is more sensitive than 1-step PCR, especially in some circumstances, such as amplification from FFPE tissue specimens. In view of the higher sensitivity of nested PCR using IS6110, special attention should be paid to the capacity of TB DNA to remain in the tissue specimen after drug therapy. Hernandez-Pando et al. (48) showed the persistence of DNA from TB in normal lung tissues during latent infection, and Salian et al. (49) suggested that healed tuberculous granulomas, which were culture and acid fast stain negative for TB, would sometimes be positive for TB DNA. This suggests that caution must be adopted in interpreting molecular data such as PCR or other similar molecular techniques because these may be too sensitive for distinguishing between active and inactive or infectious or noninfectious states of the lesion. Thus, when TB DNA is detected in lung tissue specimens, a further evaluation of other laboratory and clinical information is needed, especially before a final diagnosis of TB is performed.

4.3. Fungal infection

Fungi may cause serious morbidity in immunocompromised patients. In lung recipients lesions comprise colonization of bronchiectatic airways, cavitating pneumonia, bronchocentric granulomatosis, invasive infection with mediastinitis and infection of the airway anastomosis. Among fungi Aspergillus spp. are the most common cause of invasive fungal infection. Approximately 6% (range 3 to 15%) of lung transplant recipients develop aspergillosis (50). Early diagnosis of fungal infection is critical for optimized treatment and successful outcome. Molecular techniques appear promising tools for rapid and sensitive detection of fungal disease thus allowing a more timely initiation of antifungal therapy, when appropriate (51). However at present molecular approaches in this setting lack standardized protocols and are not able to distinguish fungal colonization from disease (52). A study by Cuenca-Estrella and colleagues (53) suggests that combining serial RT-PCR for invasive aspergillosis with galactomannan testing improves sensitivity and early diagnosis of invasive aspergillosis. Additional studies of PCR-based technologies in combination with other diagnostic tools should better clarify the feasibility and potential benefits of combination approaches to diagnosis of invasive fungal infections.

5. MOLECULAR PATHOLOGY OF OTHER LUNG LESIONS: CONTRIBUTING ROLE OF INFECTIVE AGENTS

In this paragraph a heterogeneous group of diseases (both inflammatory and neoplastic lung lesions) will be briefly covered. In these diseases infectious agents have been mostly detected by molecular methods and their etiological contributing role will be commented.

5.1. Kaposi's sarcoma, pleural effusion lymphoma, Castleman's disease and granulomatous-lymphocytic interstitial lung disease

Human herpesvirus 8 (HHV-8) has been implicated in the etiology of Kaposi's sarcoma (KS), pleural effusion lymphoma (PEL), multicentric Castleman's disease (CD) and granulomatous-lymphocytic interstitial lung disease (54). These pathological processes rarely involve lung parenchyma or pleura and occur frequently in immunocompromised patients (KS, PEL and CD) or in patients with common variable immunodeficiency—CVID—(granulomatous-lymphocytic interstitial lung disease). Unlike KS and PEL, not all CD (except for those forms arising in HIV patients) or lung lesions associated with CVID have been found to contain HHV-8 DNA thus, there is likely more
than one etiology for this somewhat diverse group of lesions. One hypothesis that would account for different pathological manifestations of infection by the same virus is that viral genes (lytic, latent forms) are differentially expressed in heterogeneous cell types (55).

As the exact frequency of HHV8 infection in these diseases is unknown, particularly in MCD and PEL, molecular investigations for this virus should be performed when these lesions are diagnosed. Moreover in such lesions the use of molecular techniques may be of help to follow the therapy efficacy. Finally, the identification of latent/replicative status could help to better understand their role in the development/progression of those diseases.

In immunocompromised patients the detection of HHV8 DNA in the bronchoalveolar lavage fluid has been considered highly specific and sensitive for the diagnosis of pulmonary Kaposis’s sarcoma. Symptoms of pulmonary Kaposis’s sarcoma include cough, dyspnea, hemoptysis and pleural pain due to pleural effusion. These symptoms as well as radiological exams (nodules, tumor masses, bronchovascular pathway thickening, and pleural effusions) are nonspecific and can mimic different lesions as those caused by other opportunistic infections. The histology is surely the gold standard for the final diagnosis of Kaposis’s sarcoma but there is a considerable risk of major bleeding after biopsy of these heavily vascularized tumors. Open lung biopsy and video-assisted thoracoscopic surgery to obtain large tissue samples are invasive procedures and rarely performed for this indication. The detection of HHV8 DNA in bronchoalveolar lavage may be considered a very useful noninvasive test to confirm suggested Kaposis’s sarcoma (56,57). As these KSHV/HHV-8-related diseases cause significant morbidity and mortality in affected patients, the identification of the virus within pathological tissue will allow for more targeted therapy. This is extremely important in lung recipients whose treatment should be changed with tapering of standard immunosuppression and/or switching to other immunosuppressive drugs and overall association of specific antiviral therapy.

5.2. Lymphomatoid granulomatosis (LYG), post-transplant proliferative disorder (PTLD) and lymphoid interstitial pneumonia (LIP)

Epstein-Barr virus (EBV) has been thought to be an important contributing factor in several lymphoproliferative lesions such as LYG, PTLD and LIP. LYG, an uncommon pulmonary lesion consisting of atypical angiocentric lymphoreticular infiltrates with variable amounts of necrosis, may occur in both immunocompetent and immunocompromised patients. PTLD occurs in 3-5% of lung recipients with frequent involvement of the allograft, often as one or multiple nodules.

Pulmonary LYG appears to be associated with EBV infection in most but not all cases. A small number of cases show no demonstrable evidence of EBV infection, and may represent a distinct form of peripheral T cell lymphoma (58). Alternatively, these cases may reflect grade 1 disease with infrequent EBV-positive cells (59).

PTLDs, morphologically characterized by lymphocyte aggregates, mainly B-lymphocytes, are also but not invariably associated with EBV. The reported incidence, however, of EBV-negative PTLDs varies widely, and it is uncertain whether they should be considered analogous to EBV-positive PTLDs and whether they have any distinctive features. A few years ago Izadi et al. demonstrated relatively better histopathological features of EBV-positive cases than EBV-negative even if the overall survival rate of EBV-positive PTLD patients was not inferior to that of the EBV-negative subjects (60).

The diagnosis of these conditions is often difficult as the physical signs, chest X-ray, and routine laboratory investigations are usually nonspecific. The diagnosis requires lung tissue samples, and rapid and precise characterization is mandatory as these lymphoproliferative disorders can be refractory to treatment and overall progress to overt lymphoma. EBV in lung samples should be sensitively investigated using molecular investigations as in situ hybridization for EBV-encoded small RNA (mRNA EBER) and the result should always be reported in a final diagnostic report. LYG can be subclassified using a grading system based on the number of EBV-positive large B-cell malignant cells and should be reported to grade LYG. This is critical in selecting appropriate management strategies (61). Although the value of non in situ recognition of EBV as DNAemia is quite discussed to monitor PTLD in lung recipients it is extremely important to note that this disorder may also occur in the absence of detectable EBV DNAemia in contrast with strong EBER positivity in lung samples (62).

LIP, a rare interstitial lung disease characterized by diffuse interstitial inflammatory cell infiltrate, is frequently associated with immunological
disorders. In immunocompromised patients (e.g. AIDS) several infective agents, in particular EBV, has been considered a potential etiological factor (63).

EBV seems to play a role also in carcinogenesis of lymphoepithelial-like large cell carcinoma (see below its tumor altered genes) only in patients from Southeast Asia, whereas in Caucasians these carcinomas are negative (64,65).

5.3. Squamous cell papilloma

Papilloma is a benign lung tumor covered by metaplastic epithelium, most often composed of squamous cells, sometimes with certain degrees of dysplasia. Koilocytes are sometimes seen, and by immunohistochemistry, ISH, or by PCR human papillomavirus (HPV) can be detected (66). A bizarre outline of nuclei is usually detected in cases of HPV 16 or 18 positive cases. Many HPV types are known; in general they are divided into oncogenic and non-oncogenic types. They all have in common to promote growth of the epithelial cells when integrated into the cell genome. Also recurrence of papillomas is common. The most important function of HPV genome is its interference with the cell cycle control proteins p53 and RB protein. Specific parts of the HPV genome induce stabilization of the p53 and RB proteins preventing apoptosis induced by genetic aberrations. Therefore the DNA repair machinery is ineffective and mitosis progress despite DNA defects and gene aberrations (67,68). Integration of oncogenic HPV types (16 and 18) will induce a rapid progression of the papilloma into invasive squamous cell carcinoma (66).

5.4. Acute and chronic rejection

Along with such direct effects as acute pneumonitis different viruses may indirectly contribute to acute rejection and the development of bronchiolitis obliterans syndrome (BOS), the major limiting factor in long-term survival after lung transplantation. CMV is one of the major viruses detected in bronchoalveolar lavage/ transbronchial biopsies and strictly correlated with graft dysfunction. The majority of studies focused on this topic have investigated viral infections using sensitive molecular tools (69). The recent CMV prophylaxis, sometimes associated with the use of CMV immunoglobulin, has reduced/delayed but did not eliminate CMV disease or CMV-complications. This is also the experience of our centre: patients treated with combined immunoprophylaxis had a significantly less incidence of CMV disease and less acute rejection than those without (Calabrese et al., unpublished data) at least in the first year after lung transplantation but it seems to have less impact on chronic dysfunction. Human herpesvirus-6, another herpesvirus frequently detected in bronchoalveolar lavage and transbronchial biopsies, has also been associated with chronic lung dysfunction (70). The overall frequency of herpesvirus-6 DNA detection in bronchoalveolar lavage was about 20%, and it was usually detected together with the other opportunistic herpesviruses CMV, herpesvirus-7 and EBV (71). However, the clinical significance of the herpesvirus-6 finding in BAL has remained unclear, probably also ascribable to the concomitance of other viral infections. Over the years the improvement of molecular tools, including real-time PCR technology, has contributed to increase the sensitivity of diagnostic procedures and new species (HMpV, coronavirus NL63 and HKU1 and bocavirus) other than CMV and CARV have also been detected in bronchoalveolar lavage or transbronchial biopsy. The results of the various papers in the literature are quite discordant and a relatively recent review which examined 34 different studies highlighted the concept that the clinical link between respiratory viruses and acute lung rejection or BOS needs to be characterized in prospective and appropriately designed cohort studies (72).

5.5. Idiopathic pulmonary fibrosis (IPF)

IPF is a progressive disease of unknown etiology characterized by a deregulated wound healing response that leads to progressive accumulation of fibroblasts and extracellular matrix which compromises tissue architecture and lung function capacity. Injury to type II alveolar epithelial cells is thought to be the key event for the initiation of the disease, and so far both genetic factors, such as mutations in telomerase (73) surfactant protein C (74) and MUC5B genes (75,76) as well as environmental components, like cigarette smoking, dust exposure, gastroesophageal reflux and viral infections have been implicated as potential initiating triggers (77). Among viral agents herpes viruses, in particular EBV, have been suggested as principal cofactors (as initiating or exacerbating agents) of fibrotic lung disease (78,79). The majority of studies investigated the presence of viruses in lung samples using sensitive molecular tools (PCR or nested-PCR) and reported a frequency of herpes viruses in IPF lungs ranging from 30 to 100%. Only a few works have demonstrated an adverse impact of herpes virus infection in IPF patients (79,80). Tsukamoto et al. reported a more rapid disease progression in EBV
positive cases. The majority of viral cases died from respiratory failure at a mean of 41 months of follow up (80). Our group has recently demonstrated a high frequency of viral infection in advanced forms of IPF and we have showed, for the first time, a different phenotype of virus-positive IPF patients. In particular virus-positive IPF cases showed more pronounced vessel remodeling and a higher mPAP, a complication that severely affects prognosis of the disease. The presence of herpessiral DNA in IPF native lungs also has a significant impact on the graft in the early post-transplant period. We have recently shown a significant higher risk of primary graft dysfunction in virus-positive IPF compared to negative cases who underwent lung transplantation (79). Molecular viral investigation in IPF lung samples, involving larger case series from different centers, could give in the future more information on the influence of these viruses on the disease.

6. FINAL REMARKS

Molecular techniques are now an important armamentarium for pathologists to establish the diagnosis of infective pneumonia. Precise characterization of infective pneumonia now requires appropriate morphological and molecular analyses and at the same time interlaboratory communication and collaboration with other specialists as pulmonologists, radiologists and microbiologists involved daily in the diagnosis and care. The contributing role of infective agents in different forms of diseases requires to be more consistently investigated particularly in those diseases with multifactorial etiology in order to weigh their role in the development/progression of the disease.

7. MOLECULAR PATHOLOGY OF LUNG TUMORS

Within the last decade many important discoveries were made in the regulation of growth, differentiation, apoptosis, and metastasis of lung cancers. These findings have dramatically changed the awareness in the oncology community about the classification of lung carcinomas. A decade ago oncologists were mainly interested to get the differentiation between small cell (SCLC) and non-small cell carcinomas (NSCLC) of the lung. With the findings of different responses for cisplatin and anti-angiogenic treatment in adenocarcinomas versus squamous cell carcinomas this simple clinical lung carcinoma classification schema was abolished. Now oncologists want to know the differentiation within NSCLC, and the near future will even increase subtyping of different NSCLC entities. In this review we will first focus on general aspects of molecular pathology in lung carcinomas and then discuss different genetic abnormalities within the different entities. These abnormalities will be ordered according to their importance such as targeted therapy and impact on outcome.

7.1. Therapy relevant molecular changes in pulmonary carcinomas

7.1.1. Non small cell lung carcinoma and angiogenesis

Angiogenesis, better neoangiogenesis is a process by which primary tumors get access to nutrients and oxygen. The process of neoangiogenesis is still not fully understood. In some cases the tumor cells themselves produce angiogenic factors such as vascular endothelial growth factors (VEGFs), in other cases these growth factors are produced by macrophages present in the tumor microenvironment (81). However, once new blood vessels (capillaries, small arteries, veins) are formed, this provides advantage for the tumor cells over their normal neighbor cells in getting better oxygen and nutrient supply. Nutrients and oxygen are not the only important factor for better growth, also purine and pyrimidine bases are essential for a dividing tumor cell (82,83). A good example how this can influence the progression from preinvasive to invasive lesions is the vascular variant of squamous cell dysplasia. It seems that the early access to blood vessels promote rapid progression into squamous cell carcinoma (84). In another preneoplasia atypical adenomatous hyperplasia (AAH) vascularization is a late event, usually at the transition from in-situ to invasive adenocarcinoma (85). This might explain why AAH can persist for several years without progression (86). In addition there seems to be a difference between mucinous and non-mucinous adenocarcinomas with respect to neoangiogenesis (87). Increased angiogenesis itself in invasive adenocarcinomas has a negative impact on survival and progression of these patients (88).

Angiogenesis is essential for the primary tumor as well as for metastasis. The secretion of VEGFs facilitates most often neoangiogenesis. Tumor blood vessels are fragile, and are prone to rupture.

In the last decade humanized antibodies, such as those against VEGF (Bevacizumab), have been developed to interfere with the neoangiogenesis
in primary as well as metastatic carcinomas (89,90). However, anti-angiogenic drugs can cause severe bleeding, especially in patients with centrally located squamous cell carcinomas. However, it is still not clear, if the reported bleeding episodes in these patients are due to the squamous histology or to the centrally located tumors, usually supported by arteries and veins arising from large branches. In addition it was reported that cavitation within the tumor is prone to hemorrhage, again something more common in central tumors located close to large blood vessels (91). Thus the use of Bevacizumab is recommended for adenocarcinomas and large cell carcinomas, but squamous cell carcinomas are excluded, since they are most often centrally located tumors.

Different strategies are being developed to inhibit the VEGF pathway, HIF signaling and hypoxia in tumor development and metastasis. In several studies the importance of the VEGF and VEGFR axis was noted for vascular invasion and metastasis, mainly involving VEGF-C and VEGFR3 (88, 92-94). Studies aiming to target this axis showed positive results in experimental settings (95-97). Bringing these targeted therapies into clinical trials is still in its infancy (98). A major problem in targeting VEGF-VEGFR is the fact that its regulation is under the major influence of the hypoxia pathway. Hypoxia is an important factor in invasion and angiogenesis, and HIF1α signaling results in the upregulation of VEGF (99,100). Thus the hypoxia pathway might constantly overrule a blockade of VEGF-VEGFR unless also HIF1α production is also inhibited (101).

7.1.3. Thymidylate synthase blocker
Pemetrexed is an inhibitor of thymidylate synthase (TS) less for the other enzymes in the thymidine cycle. Thymidine uptake is essential for rapidly dividing carcinoma cells. In tumors with low expression of TS pemetrexed can block the enzyme resulting in growth inhibition. TS expression most often is low in adenocarcinomas, but is highly expressed in many squamous cell carcinomas. Thus pemetrexed is efficient in most adenocarcinomas and not in squamous cell carcinomas (103). However, the action of pemetrexed is still not entirely clear. Thymidylate metabolism does not only rely on enzymes of the thymidylate cycle, but also needs active and passive uptake mechanisms; and thymidine uptake might also be influenced by pemetrexed (104).

7.1.4. Receptor tyrosine kinases in lung carcinomas
Receptor tyrosine kinases (RTK) are membrane-bound protein receptor composed of an external receptor domain, a transmembrane spanning portion, and an internal domain, which at its C-terminal end contains the kinase domain. The external receptor domain has a specific configuration for the binding of growth factors, where usually two molecules form homo- or heterodimer with the receptor domain. This specific binding changes the configuration of the whole receptor and leads to the activation of the kinase domain. There are two ways of activation of receptor tyrosine kinases in lung cancer: overproduction of ligands either by the tumor cell or by cells within the micro environment, such as macrophages, or activation by a mutation of the receptor gene, most often within the kinase domain. The receptor kinase itself can act also in two different ways: one is transfer of phosphorylation to transfer molecules (105,106), like GAB1 or GRB2, or the kinase splits into fragments, where one activated protein fragment translocates into the nucleus and binds to specific DNA elements and induces transcription of proteins (107). In lung cancer RTKs can be constantly activated by different mechanisms: amplification of the RTK gene, mutations of the RTK gene, gene rearrangements (translocation/inversion) with constant activation or inactivation of regulatory proteins. Another mechanism is downregulation of regulatory proteins by miRNAs, so a tumor suppressor or a negative feedback protein is not synthesized because of mRNA inactivation by miRNA (108-113).
7.1.5. TP53 the tumor suppressor gene

TP53 was one of the first tumor suppressor genes detected as being mutated in almost every cancer type. TP53 is located on chromosome 17p13-12, contains 11 exons, has two promoters (one upstream of noncoding first exon, and another within first intron) (114). The protein p53 functions as a cell cycle control, which can send cells with defective DNA directly into apoptosis (115). TP53 is either mutated or methylated in most lung carcinomas, especially in all tobacco smoke associated variants. Analysis of these types of mutations highlighted some non-functional mutations most probably due to interaction with some tobacco genotoxic compounds (116-119), whereas other mutations resulted in truncation of the protein, defect of protein degradation, and loss of function (120-122). Mutations and methylation-induced silencing is common in SCLC, squamous cell carcinomas, less frequently in adenocarcinomas (117,123,124). There is still an ongoing debate if p53 inactivation is related to a metastatic phenotype (125,126).

Next we will focus more specifically on tumor entities and what molecular profiles are known in each entity.

7.2. Non small cell lung cancer

7.2.1. Adenocarcinoma

Adenocarcinomas in highly industrialized countries are the most common lung carcinomas, with a percentage of 40% of all lung carcinomas. In addition, what was previously regarded as a single entity has become a huge diversity of carcinomas. Adenocarcinomas in never-smokers most probably represent a separate entity with different gene signatures and a slower progression rate compared to adenocarcinomas in smokers. Also gene signatures have contributed to a more heterogeneous picture. Morphologically adenocarcinomas can show a variety of patterns, which in part correlate with gene signatures, although our knowledge in this respect is still in its infancy. Adenocarcinoma is defined by the formation of papillary, micropapillary, cribriform, acinar, and solid structures, the latter with mucin synthesis and mucin-containing vacuoles in at least 10% of the tumor cells. Adenocarcinomas can be either mucinous or non-mucinous. Both will show the above-mentioned patterns. Some rare variants are fetal, colloid, and enteric adenocarcinomas. Most often a mixed pattern is seen with a predominance of at least one component. Tumor cells in adenocarcinomas can show differentiations along well-known cell types as Clara cells, type II pneumocytes, columnar cells, and goblet cells. Due to the importance of targeted therapy the exact classification of adenocarcinomas and their differentiation from other NSCLC has become a major task in pulmonary pathology. Differentiation factors are used to prove the nature of the carcinoma especially in less well-differentiated examples. A variety of useful markers have been tested, the most important ones are TTF1 and Napsin A.

7.2.1.1. EGFR

In 2004 an epidermal growth factor receptor (EGFR) mutation was detected in a patient with lung adenocarcinoma and responded to tyrosine kinase inhibitor (TKI) treatment - a new era of targeted therapy in NSCLC was invented (127,128). Mutations of EGFR have been detected in a small percentage of lung cancer patients of the Caucasian population. These are activating mutations found in exons 18, 19, 20, and 21 of the EGFR gene (kinase domain) (129). Mutations are most often found in never smokers, females, and in patients with adenocarcinoma histology. Mutations change the configuration of the kinase, which does not need anymore the ligand-based activation from the receptor domain. The receptor stays in an activated stage and constantly signals downstream. Carcinomas with this activating mutation can be growth inhibited by small receptor TKI such as Gefitinib, Erlotinib, and Afatinib. These TKIs bind either reversible or irreversible into the ATP pocket of the mutated EGFR kinase and thus inhibit phosphor-transfer to downstream molecules, thus blocking the signaling cascade (130). The most common mutations are deletions within exon 19 with a variation of 9-18 nucleotides, and a point mutation at exon 21. Other less common mutations are point mutations in exon 18, and insertions in exon 20. However, mainly within exon 20 there are also resistance mutations, the best known is T790M. This type of mutation inhibits or reverses the binding of the TKIs Gefitinib and Erlotinib and prevents the receptor blockade. The irreversible TKI Afatinib might overrule some of these resistance mutations, but more data are needed to proof this (131). For targeted therapy with TKIs tissue samples of NSCLC have to be analyzed for these mutations. Within the different subtypes of adenocarcinomas some will show a higher percentage of EGFR mutations, whereas other not. In Caucasian population adenocarcinomas with acinar or papillary pattern are up to 27% mutated, whereas mucinous adenocarcinomas are constantly negative for EGFR mutations.
Carcinomas with biphasic morphology such as adenosquamous carcinomas and mixed small cell and adenocarcinomas can show mutations but usually in a very small percentage. Another therapy approach was tested with humanized monoclonal antibodies for EGF. By competitive binding to the receptor, this antibody replaces EGF and thus inhibits transactivation of the kinase. This type of therapy seems to be especially promising in EGF-‐naïve (wild-type) adenocarcinomas and in addition also in squamous cell carcinomas (132,133).

7.2.1.2. KRAS
KRAS was one of the early-detected oncogenes in adenocarcinomas of the lung. KRAS belongs to the family of small GTPases located close to the inner cell membrane. They can be activated by tyrosine receptor kinases either membrane bound as EGFR or also by cytosolic kinases as SRC. Usually phosphorylated transfer molecules activate them as GRB2 (134-136). Once activated they can signal downstream into three major cascades: RAL-RAF, MEK-ERK, or PI3K-AKT. These different activation cascades have different effects on tumor cells, however, the exact interaction and the mechanisms, which select a specific signaling pathway are not clear (137,138). Mutations of KRAS in lung adenocarcinomas are found in the codons 12, 13, and 61. These mutations result in constant activation of KRAS and consecutively activation of the downstream cascades. KRAS in this situation does not need an upstream activation. KRAS mutations occur at an average of 30% of all pulmonary adenocarcinomas, but this percentage rises to 50% in mucinous adenocarcinomas (Geles et al. under review). In one study KRAS mutations were more frequently seen in solid adenocarcinomas (161). Whereas KRAS mutations are frequent in Caucasians, they are rare in Southeast Asian populations, opposite to the situation of the frequency of EGFR mutations (139-141).

At present time targeted therapy does not exist for patients with mutated adenocarcinomas, but there are trials going on, which aim to inhibit the downstream signaling pathway with MEK and ERK inhibitors (98).

7.2.1.3. EML4ALK1 and additional fusion partners
Inversion (erroneously called translocation) of the ALK1 kinase gene and fusion with the EML4 gene has been recently shown in patients with NSCLC, especially in solid adenocarcinomas with focal differentiation into signet ring cells. Subsequently other patterns have been associated with this type of gene rearrangement, such as micropapillary. Both genes are on chromosome 2; the chromosomal break is inversely rearranged whereby the kinase domain of ALK and EML4 are fused together. The ALK kinase thus is under the control of EML4, which results in a constant activation of the kinase. ALK, similarly to EGFR, stimulates proliferation and inhibits apoptosis. Patients with this inversion respond excellently to crizotinib treatment, which is now the second example of targeted therapy in NSCLC (142). Proof of EML4ALK1 inversion can be done with different methods. The most common is FISH where two probes (3’ and 5’) detecting the ALK gene on both sides of the breakpoint are used. In the normal situation these probes will detect the two portions close together or overlapping within the tumor nucleus. In cases of rearrangement, the probes will highlight each of the split portions of the ALK1 gene, so instead of two overlapping signals the signals split apart. In the Caucasian population EML4ALK1 rearrangement is usually found in 4-6% of NSCLC; in adenocarcinomas this might be increased to 8%. Other genes joining the ALK1 gene in the same way can replace the EML4 gene. If KIF5B joins to ALK1 the overexpression of KIF5B-ALK (111) in mammalian cells led to the activation of signal transducer and activator of transcription 3 (STAT3) and protein kinase B and enhanced cell proliferation, migration, and invasion (111). Another fusion partner recently described is ALK-KLC1 (143). These other ALK1 fusions are rare; the incidence is about 1%. Resistance mechanisms in EML4ALK rearranged lung adenocarcinomas do exist, however, the exact mechanisms are still under investigation (144,145).

7.2.1.4. ROS1
ROS1 is another kinase involved as a driver gene in adenocarcinomas of the lung (146). Usually the rearrangement of ROS1 is evaluated by two FISH probes for the 3'- and the 5' ends. Only few fusion partners have been identified so far, CD74, SLC34A2, EZR, and GOPC/FIG (147,148). This gene rearrangement has no influence on outcome, but similar to ALK1 this is usually a younger population of cancer patients (149). The incidence of ROS1 rearrangement is in the range of 1%. The function of one of the fusion genes EZR-ROS was studied in a mouse model and showed that in this experimental setting the fusion gene acted as an oncogene inducing multiple tumor nodules in mice (150). Most important patients with this type
of gene aberrations responded well to the ALK1 inhibitor crizotinib (151-153).

7.2.1.5. KIF5B and RET

KIF5B is one of the fusion partners for either ALK1 or RET. The KIF5B-RET fusion gene is caused by a pericentric inversion of 10p11.22-q11.21. This fusion gene overexpresses chimeric RET receptor tyrosine kinase, which can spontaneously induce cellular transformation. Besides KIF5B, CCDC6, and NCOA4 can form fusion genes with RET. Patients with lung adenocarcinomas with RET fusion gene had more poorly differentiated tumors, are younger, and more often never-smokers. Solid adenocarcinomas predominate, tumors are smaller but lymph node incidence is higher. The incidence of RET fusion is about in 1% of NSCLCs and almost 2% of adenocarcinomas (154-156).

7.2.1.6. MET

MET is another receptor tyrosine kinase bound to cell membranes in NSCLC. The ligand for MET is HGF, originally found in hepatic carcinomas. This receptor came into consideration in NSCLC because amplification of MET or alternatively upregulation of HGF was identified as a mechanism of the resistance in EGFR mutated adenocarcinomas (109,157). A search for the role of MET in other NSCLC excluding EGFR mutated adenocarcinomas showed that MET amplification was rare, but upregulation of MET a common event: approximately 20% of NSCLC including adenocarcinomas and squamous cell carcinomas showed high protein expression, but only 2% MET amplification (Popper et al., unpublished data). Clinical studies are in progress to evaluate the possibility to interfere with MET signaling using monoclonal antibodies. Other studies use small molecule inhibitors for MET. Since MET expression is common in EGFR mutated adenocarcinomas these studies aim to inhibit both EGFR and MET signaling pathways (158).

7.2.1.7. Other genes

Histone acetylases and deacetylases (HAT, HDAC) regulate the access of the DNA by methylation and demethylation. Thus these enzymes are important for DNA silencing (159,160). In addition heavily methylated DNA tends to switch into a supercoiled form, which cannot be read the transcription machinery. Histones themselves are in addition important for the correct positioning of the DNA, fixing the DNA to specific areas of the nuclear membrane (161,162). Attempts to interfere with this system were made quite a while ago, but the results were not convincing, probably because there are several types of HATs and HDACs with different functions. Recently treatment with HDAC inhibitors in combination with other drugs has shown promising results resulting in apoptosis and tumor cell necrosis (163-165).

In a study focusing on molecular alterations in pulmonary adenocarcinomas many additional genes were identified: well known are losses of one allele of the tumor suppressor PTEN in 9%, often associated with upregulation of PIK3CA, however PIK3CA mutations were also detected in 5%. Two other genes mutated in 3% and 2% were identified as STK11 and BRAF, respectively (166). Interestingly these gene alterations could be sorted by smoking habits to either smokers (STK11) or non-smokers (PIK3CA). In addition squamous cell carcinoma morphology was associated with PTEN, STK11, and PIK3CA (166).

In a study analyzing Korean lung cancer patients by transcriptome sequencing the authors identified the well known candidates EGFR, KRAS, NRAS, BRAF, PIK3CA, MET, and CTNNB1, but also new driver mutations such as LMTK2, ARID1A, NOTCH2, and SMARCA4 (167). Besides these mutated genes also fusion genes were detected as ALK, RET, ROS1, and new ones as FGFR2, AXL, and PDGFRA. We also found an association between lymph node metastasis and somatic mutations in TP53. Elevated levels of insulin-like growth factor (IGF)-II are associated with a poor prognosis in human pulmonary adenocarcinoma. Moorehead et al. succeeded in establishing pulmonary adenocarcinoma in mice by transgenic overexpression of IGF-II in lung epithelium. These tumors expressed TTF-1, SP-B and proSP-C. Activation of IGF-IR resulted in the downstream activation of either the ERK1/ERK2 or p38 MAPK pathways (168). Within this IGF/IGFR system also binding proteins play a prominent role: IGFBP3 expression resulted in up-regulation of VEGF and HIIF1, underlying the importance in neoangiogenesis and consequently in tumor growth and invasion (169). In contrast IGFBP1 seems to act like a tumor suppressor decreasing colony formation of cell cultures and increasing apoptosis. IGFBP1 has been found methylated in pulmonary adenocarcinomas (170). The IGFR1 pathway is also involved in resistance mechanisms in EGFR mutated adenocarcinomas (171). Treatment with monoclonal antibodies for IGFR1 also occurs in clinical studies (172,173).
7.2.2. Squamous cell carcinoma

Squamous cell carcinoma is defined by a plate-like layering of cells, keratinization of at least single cells, intercellular gaps and bridges (represented by desmosomes and hemidesmosomes), and positive staining for high molecular weight cytokeratins (CK 3/5, 13/14). There are some morphological variants as small cell and basaloid, but these have not been associated with gene signatures and therefore are only important in diagnostics. The incidence of squamous cell carcinoma has dropped in the last three decades from a major entity representing 35% of lung carcinomas to around 17%. One of the major reasons is the shift from filter-less to filter cigarettes. This has resulted in the reduction of particle-bound carcinogens and increase of vaporized carcinogens, which more easily reach the bronchioalveolar terminal unit, inducing mainly adenocarcinomas. In the past squamous cell carcinoma was mainly a diagnosis required to exclude several therapeutic options in the clinic: no pemetrexed therapy, no antiangiogenic drugs, less responsiveness to cisplatin treatment. However, this has changed within the last three years.

7.2.2.1. FGFR1

Fibroblast growth factor receptor 1 was identified being amplified in about 20% of squamous cell carcinomas (174 and Popper et al., unpublished data). In experimental studies as well as in ongoing clinical trials it was found that only amplification, proven by ISH identified patients, that responded to small molecule inhibitor treatment (175) (and unpublished communication from R. Thomas, Cologne, Germany).

7.2.2.2. DDR2 and FGFR2

DDR2 and FGFR2 mutations are found exclusively in squamous cell carcinomas, however, in a small percentage, 4% and 2%, respectively (166). For FGFR2 multikinase inhibitors might be an option for specific treatment (176,177).

7.2.2.3. SOX2 amplification

SOX2 gene located on chromosome 3q26.3. is a factor for the maintenance of stem cell like properties in lung cancer cells (178). SOX2 amplification has been reported to be specifically associated with squamous cell carcinoma morphology (179-181). However, other investigations have also claimed an importance for small cell carcinomas and adenocarcinomas (178,182). Amplification in squamous cell carcinoma was associated with better prognosis (183) whereas with poor prognosis in adenocarcinomas and SCLC. So far no specific therapies do exist for patients with this genetic abnormality.

7.2.2.4. PTEN mutation-deletion

PTEN deletions are quite common in NSCLC, usually associated with the subsequent upregulation of PI3K and downstream activation of the AKT pathway (184). In the past therapies were conducted with inhibitors of mTOR, but failed due to the subsequent upregulation of a negative feedback loop of mTOR, which in turn activates AKT and this time results in a circumvention of mTOR via other pathways (185-187). Recent works, however, have shown a benefit of mTOR inhibition, when applied in the right context (188,189). PTEN mutations are rare, but can be found more often in squamous cell carcinomas (166). If patients with this genetic abnormality can also be treated by a combined modality is still an unsolved issue.

7.2.2.5. PDGFRA amplification

Amplification of the PDGFR alpha was predominantly found in squamous cell carcinomas (190). Although this is not a frequent event in these carcinomas, specific inhibitors have shown a growth inhibiting effect in cell lines and might be considered in patient treatment (191).

7.2.2.6. CDKN2A (p16) mutation, deletion, and methylation

Another uncommon genetic modification is found in the CDKN2A gene coding for the p16 protein. P16 is regarded as a tumor suppressor protein and is involved in cell cycle regulation in many pulmonary carcinomas including squamous cell carcinoma and adenocarcinoma (192-194). It closely interacts with Rb and p53 proteins.

7.2.2.7. NOTCH1 mutation

NOTCH1 regulates cell specification and homeostasis of stem cell compartments, and it is counteracted by the cell fate determinant Numb. NOTCH signaling is altered in approximately one third of NSCLCs. Loss of Numb expression results in increased NOTCH activity; in a smaller fraction of cases gain-of-function mutations of the NOTCH1 gene are present. Inhibitors of Notch can selectively kill epithelial cell cultures harboring constitutive activation of the NOTCH pathway (195). In a subsequent study NOTCH1 and 2 frame shift and nonsense mutations were identified in pulmonary squamous cell carcinomas (196). More importantly
**NOTCH1** has an important role in carcinogenesis by suppressing p53-mediated apoptosis and regulating the stability of the p53 protein. **NOTCH1** also plays an important role in **KRAS** induced mouse adenocarcinoma models (197).

### 7.2.2.8. REL amplification

In a mouse model it was shown that different downstream activation of RAS pathways is necessary to induce an invasive and more important angioinvasive phenotype. Only the combined activation of the **PI3K, RAS-MAPK-ERK**, and the **RAL** and **REL** pathway could induce an aggressive phenotype (138). Whereas **REL-A** seems to be exclusively expressed in squamous cell lung carcinoma, **REL-B** was shown to be processed in NSCLC cell lines. **REL-B** was shown to suppress the expression of β1-integrin and thus prevented adherence of the carcinoma cell (198).

### 7.2.3. Large cell carcinoma

Large cell carcinoma is defined by large cells (> 25 μm) devoid of any cytoplasmic differentiation, and large vesicular nuclei. They have a well-ordered solid structure and ultrastructural tumor cells show hemidesmosomes, tight junctions, intracytoplasmic vacuoles with microvilli, and ill-formed cilia. This clearly fits into the concept of a carcinoma, at the doorstep of adenocarcinoma and squamous cell carcinoma differentiation. Large cell carcinoma numbers have dramatically decreased due to the use of immunohistochemistry for differentiating. Using **TTF1** low-molecular cytokeratins, as well as p63 and cytokeratin 5/6 most cases of large cell carcinoma were either shifted into adenocarcinoma or squamous cell carcinoma, respectively (199). These recent changes make an evaluation of genetic aberrations in large cell carcinoma quite difficult, since genetic studies were based on previous classifications.

Not surprisingly **EGFR** mutations, **MET** amplifications, and **EML4ALK** fusions have been reported in large cell carcinoma (200). **LKB1** a gene mutated in a small percentage of adenocarcinomas was also shown in squamous and large cell carcinomas (201). **LKB1**, also known as **STK11**, is involved in the negative regulation of **mTOR** and closely cooperates with **TSC1** and 2 genes (202).

In contrast to large cell carcinoma, which is negatively defined by exclusion criteria, the variants are positively defined.

#### 7.2.3.1. Large cell carcinoma with rhabdoid phenotype

**Large cell carcinoma with rhabdoid phenotype** is characterized by a solid growth pattern, often overlaid by a reactive proliferation of pneumocytes, which can give these tumors a pseudoalveolar pattern and a pseudo-composition of two cell populations. Within the cytoplasm of the tumor cells eosinophilic inclusion bodies can be found, similar to those seen in rhabdomyosarcomas. These inclusion bodies are stained by eosin, are negative for striated muscle markers, but positive for vimentin. The production of vimentin filaments, which seem to have no function because of package into a cytoplasmic vacuole, is the only known abnormality for this tumor type. In a single study **KRAS** mutations were found in some cases of this tumor type (199).

Sheets of undifferentiated tumor cells embedded in a lymphocyte rich stroma characterize *lymphoepithelial-like large cell carcinoma*. The carcinoma cells are positive for cytokeratins 13/14 and the lymphocytes in most cases are B-lymphocytes. Only one study looked up genetic changes in this tumor entity. They found unusual mutations in **TP53** at exon 8, and **EGFR** mutations in few patients within their large series. These were exon 21 (not L858R) and exon 18 and 20 mutations (203).

#### 7.2.3.2. Pulmonary clear cell carcinoma

**Pulmonary clear cell carcinoma** is another rare variant of large cell carcinoma, defined by abundant glycogen in the cytoplasm of the tumor cells. Tissue processing usually dissolves glycogen, thus leaving the impression of a clear/empty cytoplasm. Only one study examined molecular changes in this tumor type and found predominantly **KRAS** mutations (199).

### 7.3. The neuroendocrine tumors

#### 7.3.1. Small cell neuroendocrine carcinoma

Small cell lung carcinoma (SCLC) is defined by nuclear size of 16-23 μm (not so small!), dark stained nuclei (mainly composed of heterochromatin), inconspicuous or lacking nucleoli, small cytoplasmic rim, often invisible in light microscopy, and fragile nuclei. SCLC is regularly positive for the neuroendocrine markers **NCAM** and **synaptophysin**, but most often negative for **chromogranin A**. The best marker is **NCAM** with a strong membranous staining. SCLC is positive for low molecular weight cytokeratins. SCLC produces hormones, such as adrenocorticotropin (ACTH), but also substances interfering with the blood coagulation
system. In contrast to carcinoids SCLC more often are positive for heteropic hormones (i.e. hormones usually not found in adult lung). In our experience a positive reaction for gastrin-releasing peptide (GRP) and ACTH is most often seen. The secretion of ACTH can cause Cushing syndrome. Some of the hormones especially GRP act as an autocrine loop: the peptide is produced by the cancer cells, released and bind back to their respective membrane bound receptors, which themselves signal back into the nucleus with a growth stimulation (204).

Genetic abnormalities in SCLC are quite common, usually over 50% of the SCLC chromosomes are affected (205,206). These many genomic alterations made a search for driver gene mutations/alterations complicated, which consequently resulted, that besides classical chemotherapy, no targeted therapy has emerged up to now. Some genetic alterations are known for a long time, however, not resulting in a therapeutic intervention strategy. Two genetic alterations have been long known, RB loss or mutation, and TP53 mutation. Since both genes are involved in cell cycle checkpoint controls, this might explain the high numbers of genetic alterations (207-209). Other genes involved in SCLC are the tumor suppressor FHIT, RASSF1, both on chromosome 3p, RARbeta, and Myc genes (209). RASSF1 mRNA expression was lost in all tested SCLC cell lines, whereas its promoter was methylated in some NSCLC cell lines (210). In SCLC also apoptotic and immunogenic mechanisms seem to be inactivated. In a study by Senderowicz Fasl was overexpressed in almost all SCLC cases examined. The ratio of Fas/FasL was decreased. The authors concluded, that FasL overexpression in the context of Fas downregulation might allow tumor cells to induce paracrine killing of cytotoxic T cells (211). Since the PI3K-Akt signaling pathway is activated in almost all cases of SCLC, this system might also be associated with inhibition of apoptosis via upregulation of TNFRSF4, DAD1, BCL2L1, and BCL2L2, and with chemoresistance (212,213). These data demonstrate that several systems are involved in SCLC growth, survival, and resistance to chemotherapy. ASH1 was identified as the gene responsible for the neuroendocrine phenotype in both high-grade carcinomas (214). Together with other genes (ATOH1, NEUROD1 and 4) involved in neurogenic differentiation, they are also expressed in NSCLC with neuroendocrine phenotype. As SCLC, these NSCLC cases expressed mRNA for dopa decarboxylase and stained positively for neuroendocrine markers (215). ASH1 seems to be an early differentiation gene in the developing lung. In embryonic development ASH1 was found in neuroepithelial bodies and solitary neuroendocrine cells, but vanishes with maturation of the lung. Therefore ASH1 might be an early program for neuroendocrine cell differentiation (216). If there is another function of ASH1 it is not entirely clear. ASH1 seems to repress tumor suppressor such as DKK1 and 3, which are regulators of the Wnt-β catenin pathway. ASH1 also inactivates E-cadherin and integrinβ1 by de-acetylation and methylation of the DKK1 and E-cadherin promoters (217). In an animal lung cancer model the expression of ASH1 enhances the carcinogenic effect of SV40 large T antigen, suggesting that ASH1 might cooperate with pRB (218).

7.3.2. Large cell neuroendocrine carcinoma (LNEC)

LNEC is defined by a neuroendocrine pattern, e.g. rosettes, trabecules. On low power they look similar to carcinoids, but on higher magnification abundant mitoses are obvious. The prognosis of LNEC is similar to that of SCLC, both are grouped as the high-grade neuroendocrine carcinomas.

Genetic analysis of LNEC showed similar alterations as found in SCLC. However, allelic losses at 5q and abnormalities in the p16 gene may differentiate large cell neuroendocrine carcinoma from SCLC (219). Another difference between both high-grade neuroendocrine carcinomas is seen at chromosome 3q: gains of 3q are frequently seen in SCLC, whereas were absent in LNEC. However, gains of 6p were frequent in LNEC; deletions within 10q, 16q, and 17p were more common in SCLC (220). ASH1 mRNA was found higher in SCLC, whereas its counteracting gene HES1 was more frequently expressed in LNEC (221).

7.3.3. Carcinoids

Typical carcinoid is defined by neuroendocrine structures, such as rosettes, trabecules, and solid nests, 0 or 1 mitosis per 2 mm², and absence of necrosis. Atypical carcinoid is defined by 2-10 mitoses per 2 mm², and/or presence of necrosis, and again neuroendocrine structures. In both carcinoids there is an invasive growth into the lung, and lymphatic and blood vessel invasion can be found in some cases. Some carcinoids can metastasize, but so far there are no predictive markers for the biological behavior.

Those carcinoids, which have more than two losses on distal chromosome 11q, and those with multiple chromosomal losses (<10), also show...
a worse outcome. Based on genetic studies it can be speculated, that the 140 kDa isoform precursor of NCAM, Zinc-finger protein-like 1, and sorting Nexin 15 might be involved in the genesis of carcinoids (222).

7.4. Other rare lung carcinomas

7.4.1. Salivary gland type carcinomas

Salivary gland tumors can occur in the lung, usually in a central location. Within the spectrum are mucoepidermoid carcinomas, adenoid cystic carcinomas, and epithelial-myoepithelial carcinomas. These are rare carcinomas with a wide range of affected ages, from children as early as 3 years of age, as well as in elderly patients.

7.4.1.1. Mucoepidermoid carcinoma

Due to their central location they may grow as a polypoid mass occluding large bronchi and thus causing obstruction, with often poststenotic bronchopneumonia. In low-grade carcinomas cystic and solid areas are found. Within the glandular areas squamous epithelium is interspersed, the tumor cells are usually non-keratinizing. In high-grade carcinomas the distinction from adenosquamous carcinoma might sometimes be impossible, due to overlapping features. The proof is the mixture of squamous and mucin-producing columnar cells within a gland, endobronchial growth, and absence of keratin pearls and an in-situ component. The prognosis of high-grade mucoepidermoid carcinomas is similar to other NSCLC.

*EGFR* was studied in mucoepidermoid carcinomas of the lung. Protein expression was frequently found, whereas mutations in exon 18-21 were absent. Polysomy of the *EGFR* was seen in a small percentage of cases (223). In another study the authors reported *EGFR* mutations and also a response to tyrosine kinase inhibitor treatment. One of the patients in this study showed a response to treatment without alterations of the *EGFR* (224). In a clinical study growth of adenoid cystic carcinoma could be inhibited by a multikinase inhibitor (230). However, in a subsequent clinical trial this inhibition could not be confirmed (231).

7.4.1.2. Adenoid cystic carcinoma

Similar to the salivary counterpart adenoid cystic carcinoma of the bronchus is a slowly growing tumor with late lymph node and distant organ involvement. However, recurrence is frequent. They occur in the trachea and the large bronchi as far as normal bronchial glands are found. The tumor forms pseudotubules filled with mucin-like material, which is composed of matrix proteins of the basal lamina as collagen type 4 and fibronectin.

In an experimental model of adenoid cystic carcinoma it was shown that dual inhibition of *EGFR* and *VEGFR2* resulted in apoptosis, decreased angiogenesis and metastasis (226). In an array-CGH study a gain was found on chromosome 4q12, which contains a cluster of receptor tyrosine kinases such as *KIT, PDGFRA* (196), however, in another study looking at this area *KIT* mutations or amplifications were not found (227). Also mutations of *EGFR* could not be demonstrated in adenoid cystic carcinomas, but upregulation of the protein was a constant feature (223,228). In another study experimental inhibition phospho-ERK1/2 resulted in inhibition of tumor cell growth and metastasis (229) and in a clinical study growth of adenoid cystic carcinoma could be inhibited by a multikinase inhibitor (230). Recently new investigations came up with genetic aberrations in these carcinomas. The *MYB-NFIB* fusion resulting in overexpression of *MYB* was found in the majority of these carcinomas (232). Copy number alterations including down-regulated suppressor genes were in addition associated with behavior of these carcinomas (232). Newly identified mutations in adenoid cystic carcinomas, some of which might lead to a targeted therapy are *FGF-IGF-Pi3K* mutations and mutations in *NOTCH1/2* signaling regulator *SPEN*, which might be a new cancer gene in these types of tumors, and finally within a subset of these carcinomas also *FGFR2* mutations (233,234).

Another rare salivary gland tumor is acinus cell carcinoma. Uniform tumor cells showing acidophilic/azurophilic granules in their cytoplasm characterize it. So far no specific genetic abnormality has been reported in pulmonary variants.

7.4.2. Sarcomatoid carcinomas

The sarcomatoid carcinomas are a group of carcinomas with sarcomatoid features. This group is composed of pleomorphic carcinoma, spindle and giant cell carcinoma, pulmonary blastoma, and carcinosarcoma. Pleomorphic carcinoma is defined as a carcinoma with either a spindle or giant cell component and any other NSCLC component.
Pulmonary blastoma is composed of a fetal type adenocarcinoma and an embryonic type of stroma. Most often this stroma component is benign, however, in rare cases the stroma can exhibit atypia and even malignancy. Finally carcinosarcoma is composed of any type of NSCLC with a sarcoma with osseous, rhabdomyoblastic, or chondroblastic differentiation.

Few molecular studies have focused on these tumor types. In the study by Pelosi the epithelial components of sarcomatoid carcinomas showed expression of epithelial markers and cell cycle inhibitors P21WAF1 and P27KIP1, and the loss of FHIT, whereas the sarcomatoid components expressed mesenchymal markers and differently reacted for molecules involved in cell differentiation, cell cycle control, and tumor cell growth and motility (235). In another study the mechanisms of epithelial-mesenchymal transition was explored. The classical pathways NOTCH1, SNAIL, and the WNT-pathway could be excluded, but a possible alternative pathway including JUN-VIMENTIN-FASCIN was found (236). Looking up EGFR and KRAS mutations in sarcomatoid carcinomas no mutations were found for EGFR, but KRAS in almost half of the cases. However, EGFR was constantly overexpressed at the protein level, which might suggest the possibility of an EGF antibody treatment (237).

8. MOLECULAR PATHOLOGY OF PRE-NEOPLASTIC LESIONS

Actually our knowledge is very small on preneoplastic lung lesions: for a few carcinomas the preneoplastic lesion is known, but for most carcinomas discussed above we know nothing. And most importantly even for those well known lesions we cannot predict, if these lesions will progress into carcinoma or not. The factor(s) responsible for progression and invasion are unknown as well as are the ways for their sensitive detection. From our current understanding the neoplastic transformation has some important requirements, which usually precede morphological changes: nutrition, nucleotides for DNA replication, and oxygen for metabolism. Preneoplastic cells need increased access to nutrients for building up an energy reserve for the increase of cell divisions, they need to get access to higher levels of purine and pyrimidine bases to facilitate their increased DNA replications during mitosis, and for an increased metabolism also an increase of oxygen might be required. Increased oxygen consumption, however, is controversially discussed: hypoxia seems to be critical for invasion and metastasis, so probably increased oxygen and oxidative metabolism might be required only in the early intraepithelial expansion of the preneoplastic cells, whereas glycolysis seems more common in invasive carcinomas (238).

8.1. Hyperplasia of goblet cells and squamous metaplasia/dysplasia

For squamous cell carcinomas the preneoplastic lesion has been known for a while. In the large bronchi there is a protection program for toxin/carcinogen exposure, which starts with goblet cell hyperplasia and proceeds to squamous cell metaplasia, and further on to squamous cell dysplasia or intraepithelial neoplasia. A vascular variant of dysplasia has been described characterized by an ingrowth of capillaries into the squamous epithelium. Atypia and mitosis might be mild or absent, respectively, however this represents a rapid progressive lesion (84) most probably due to the expression and release of VEGF (Popper et al., unpublished data).

A few factors influencing this progression have been identified, such as a p63 splice variant, amplification of PI3KCA, increased NFkB p65 nuclear expression, gradual increases of caspase-8, STAT 5, and p70s6K or decrease of E-cadherin (239-242) Angiogenic stimuli seem to play a role in progression, since it is upregulated in rapid progressive squamous cell dysplasia with prominent vascular intraepithelial growth pattern (84). However, the interrelation of genes upregulated and downregulated has not been elucidated, so we are presently limited to single studies looking for different genetic abnormalities.

8.2. Atypical adenomatous hyperplasia and bronchial columnar cell dysplasia

They are two preneoplastic lesions confined to the alveolar and bronchiolar periphery, respectively. Atypical alveolar hyperplasia is visible already at low power magnification. The normal epithelium is replaced by pneumocyte type II like atypical cells. Between the cells, lined up as a single row, there are usually gaps left. When these gaps are lost, and cell pile up close to each other, or form cell papillae without stroma this already fulfills the criteria of adenocarcinoma in-situ. Atypical adenomatous hyperplasia is regarded as the precursor for some non-mucinous adenocarcinomas, especially acinar and papillary types.
Genomic aberrations were found to increase from atypical adenomatous hyperplasia to adenocarcinoma in-situ and invasive adenocarcinoma; the chromosomes involved in the atypical adenomatous hyperplasia lesions were found to be present in the high grade lesions, suggesting an atypical adenomatous hyperplasia to adenocarcinoma in-situ to invasive adenocarcinoma sequence (243,244). KRAS mutations were detected in one third of atypical adenomatous hyperplasia studied and seem to be an early event in carcinogenesis (245). A loss of either TSC1 or 2 genes was another finding in well differentiated adenocarcinomas and their associated atypical adenomatous hyperplasia lesions (246,247). EGFR mutations as well as amplifications could not be detected in atypical adenomatous hyperplasia, but were found in adjacent well differentiated adenocarcinomas, and less frequently in less differentiated ones (248). In another study comparing KRAS and EGFR mutations in atypical adenomatous hyperplasia, adenocarcinoma in-situ, and invasive adenocarcinomas the percentage of KRAS mutations decreased from atypical adenomatous hyperplasia to adenocarcinoma in-situ to invasive adenocarcinomas, whereas EGFR mutations increased from atypical adenomatous hyperplasia to adenocarcinoma in-situ and to invasive adenocarcinomas. This study concluded that two different pathways of atypical adenomatous hyperplasia exist, one driven by KRAS, the other by EGFR mutations, respectively (249).

In contrast to atypical adenomatous hyperplasia bronchial columnar cell dysplasia can only be identified at high magnification. In bronchial columnar cell dysplasia atypical cells gradually replace normal bronchial epithelium. In early stages of bronchial columnar cell dysplasia a monomorphic proliferation of cells replace these differentiated cells and gradually expand up to the surface. If completely replaced, more atypia is recognized.

In a study of genetic aberrations Ullmann found an increase of genetic alterations from 2.6.
in bronchial columnar cell dysplasia to 14.7 in the concomitant adenocarcinoma. Unbalanced numerical aberrations were losses of 3p, 9, 13, 14 and gains of 1q, 17, 19q and 20q (250). Another study demonstrated loss of *P16INK4a* protein in 70% of cases. p53 accumulation was found in 26% of the cases (251).

Atypical goblet cell hyperplasia is much more difficult to recognize: the nuclei are compressed at the cell border and the chromatin structure is invisible. Atypical, signet-ring-like cells replace the normal epithelium resulting in a monotonous pattern. Atypical goblet cell hyperplasia might give rise to the different mucinous adenocarcinomas of the lung. Atypical goblet cell hyperplasia was studied in cystic pulmonary adenomatous malformation. In this study, chromosomal gains on chromosomes 2 and 4 were found as the major genetic alteration (252). The same genetic alterations were demonstrated in three associated adenocarcinomas of children. Thus most probably oncogenes located on these chromosomes are involved in carcinogenesis.
8.3. Neuroendocrine cell hyperplasia

It is divided into neuroendocrine hyperplasia associated with fibrosis and bronchiectasis or in the vicinity of a carcinoid, and diffuse neuroendocrine hyperplasia without known cause; in addition a nodular proliferation is described and finally there is a tumorlet, which is different from carcinoid by size and separation of tumor cell groups by fibrous bands. Tumorlets as well as diffuse neuroendocrine hyperplasias are often found in the vicinity of carcinoids, and therefore it is speculated that they may represent preneoplastic lesions for carcinoids. A few studies focused on molecular changes in these lesions. Since neuroendocrine lesions and low-grade tumors are usually rich in capillaries, one study focused on angiogenetic factors.

VEGF was found highly expressed in diffuse proliferations, but there were no correlations with progression, but rather an association with fibrosis (253). N-Cadherin was found in a high proportion of diffuse hyperplasias and carcinoids, but less in SCLC and LCNEC. In carcinoids N-Cadherin expression was associated with negative lymphnode status (254).

9. MALIGNANT TUMORS OF CHILDHOOD

9.1. Pleuropulmonary blastoma

Pleuropulmonary blastoma is a malignant mesenchymal tumor, which can arise in the pleura, the lung, or both. It can present as a predominant cystic, a mixed cystic and solid, or a pure solid lesion, which is also reflected by increased aggressive behavior. The tumor cells form layers of immature small cells (so called germinal layer) with relatively large nuclei and dense chromatin. In addition interspersed giant cells with rhabdomyoblast differentiation and areas of chondrosarcoma are usually seen (255).

Genetically gains of large parts or whole chromosome 8 are often found in pleuropulmonary blastoma (256,257). A few other aberrations have been reported: losses on 6q13-pter, 10pter-p13, 10q22-qter, and 20p13, and gains of chromosomes 2 and 8p11-p12 (258). In some reports mutations of the TP53 gene were described (259). Recently DICER 1 syndrome has been linked to pleuropulmonary blastoma: DICER is a protein involved in the processing of small inhibitory microRNAs. A mutation has been reported in several families and this was
linked to the development of pleuropulmonary blastoma and other childhood tumors (260).

9.2. Congenital myofibroblastic tumor

Congenital myofibroblastic tumor is another rare childhood tumor composed of proliferating myofibroblastic cells, sometimes with marked cellularity and mitotic activity. The tumor cells proliferate within the interstitium and peribroncholar area, and thus cause bronchiolar obstruction. They can behave biologically like malignant tumors (261). A single genetic analysis was reported, under the other name of the tumor, namely childhood leiomyosarcoma. The authors reported gains of chromosomes 2 and 11, and losses of chromosomes 9, 19, 20, and 22 (262).

10. FINAL REMARKS

Several other topics, which are more general and not organ based in the development of cancer, are not discussed herein, such as the stem cell theory in cancer development and metastasis. Another aspect is invasion and metastasis of lung cancer. Up to now the picture in lung cancer is unclear: it seems there is no single metastasis gene for the different entities, and also the mechanisms of invasion do not follow a general rule for each carcinoma type. Survival in the blood stream and adherence on endothelial venules at the metastatic site is diverse, as well as the preference of metastasis. Some carcinomas prefer brain, others bone marrow, and others liver and adrenal glands as their primary metastatic site outside the thorax. Moreover it seems there are different genes associated with this metastatic preference. The reader is therefore referred to special reviews on these subjects.

During the last few years a high number of genes have been identified as potential biomarkers of different lung tumors. Some of these are routinely used in targeted therapy approaches. Morphology remains the gold standard for the diagnosis. New technologies, as molecular tools, should always appropriately applied and the pathologist plays a crucial role both in the selection of the most representative tissue and in the correlation between molecular signature and histotypes.

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