Lung Cancer

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Introduction

Lung cancer is worldwide the leading cause of cancer-related death [1–3]. Over 75% of the newly detected lung cancer patients have at the time of the diagnosis already distal or regional metastases [4]. Malignant pleural effusions represent advanced metastatic disease. Such metastatic involvement of the serosal cavities occurs in 15–26% of the cases [5, 6], often being the first clinical manifestation of a malignant process. With appropriate adjuvant analyses, the serous effusions can provide the necessary diagnostic information for choice of therapy. This is in particular true also for predictive analyses of tumor genetics, the unfixed effusions, in fact, being more suitable for analyses of nucleic acids than formalin-fixed paraffin-embedded tissue.

Although most lung cancers supposedly develop from the same epithelium lining the bronchial or bronchiolar-alveolar walls, the histological appearances of these tumor tissues vary considerably. Non-small-cell lung cancer (NSCLC) accounts for about 80% of all lung cancer cases, whereas the remaining 20% corresponds to small-cell lung carcinoma (SCLC). Traditionally, NSCLCs are further categorized into tree main groups: squamous cell carcinoma (SCC), adenocarcinoma (AC), and large-cell carcinoma (LCC). The WHO subdivides each of these further into subgroups, and together with some less common tumor types, such as tumors from mucosal glands and sarcomas, the classification describes some 50 different histological growth patterns of malignant lung tumors [7]. When a malignant condition is diagnosed in a serous cavity, however, molecular characterization is clinically more important, and the histogenetic classification is often limited to the main tumor phenotypes. Although therapy often has profound effects on the subgroup of patients with targetable mutations, only limited improvements have been achieved in the overall 5-year survival of lung cancer when treated with chemotherapy, which remains stably around 15% [8]. This might at least partly be attributed to late detection, morphological and biological heterogeneity, and frequent occurrence of primary or secondary resistance to chemotherapy.

Histogenetic Classification of Lung Cancers

Lung AC shows the largest variability within the tumor group. By light microscopy these glandular tumors sometimes form mucins that can be demonstrated by histochemistry and immunohistochemistry. Correspondingly various types of secretory granules can be seen by electron microscopy (Fig. 8.1a). Glandular differentiation of lung cancer is often associated with the expression of cytokeratins (CKs) 7 and 18 and mucin type 1 (MUC1). Napsin A and TTF-1 are also typically expressed in most lung ACs [9]. These epitopes are, however, not too infrequently expressed also in the other types of bronchogenic carcinoma and serve better to distinguish primary lung cancers from metastases. Particular forms of lung ACs are those originating from the peripheral parts of the bronchial tree. Based on the latest WHO classification [7], however, the term bronchioloalveolar carcinoma (BAC) should be restricted to an in situ condition with lepidic spread along pre-existing alveolar walls, respecting the basement membrane. Ultrastructurally, tumors with pneumocytic phenotype contain typical surfactant multilamellar bodies. These structures can also be seen in tumors with unequivocal infiltrative growth (Fig. 8.2), and surfactant can be recognized immunohistochemically. Still, invasive cancers with bronchioloalveolar cell phenotype are just classified as ACs. Following the WHO classification, lung ACs can be categorized into altogether 14 subgroups.

SCCs constitute a similarly large group. The cells are often keratinized and form intercellular bridges—the result of desmosome junctions becoming visible because of shrinkage during preparation. Ultrastructurally these cells show abundant desmosomes with tonofilaments formed by coarse

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B. Davidson et al. (eds.), *Serous Effusions*, https://doi.org/10.1007/978-3-319-76478-8_8

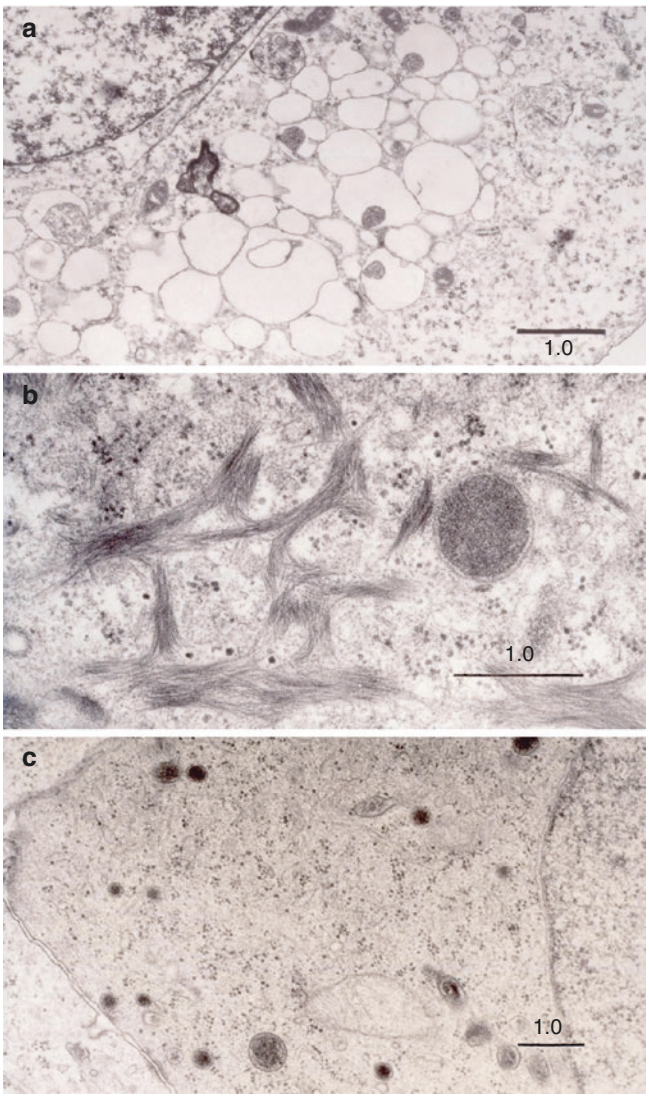

Fig. 8.1 Three pleural effusions with carcinoma cells, showing small-cell morphology in light microscopy. The presence of secretory granules, shown by electron microscopy (**a**) reveals, however, that this tumor in fact is an adenocarcinoma, while the abundant tonofilaments (**b**) demonstrates the epidermoid phenotype of a squamous cell carcinoma. Cells from the third tumor (**c**) contain numerous neurosecretory granules verifying the true neuroendocrine phenotype of a small-cell lung carcinoma. Bar 1 μm

bundles of CKs (Fig. 8.1b). Immunohistochemically, they can be recognized by expression of CKs 5 and 17 together with p63. According to the WHO classification, five different patterns of squamous differentiation can be recognized.

SCLC constitutes the third common form of lung cancer. Lung cancers with small-cell morphology are heterogeneous and show variable ultrastructure and immunophenotype, some cases merely being poorly differentiated SCCs and others ACs. The tumor cells of the particular SCLC group contain neurosecretory granules (Fig. 8.1c), which also can

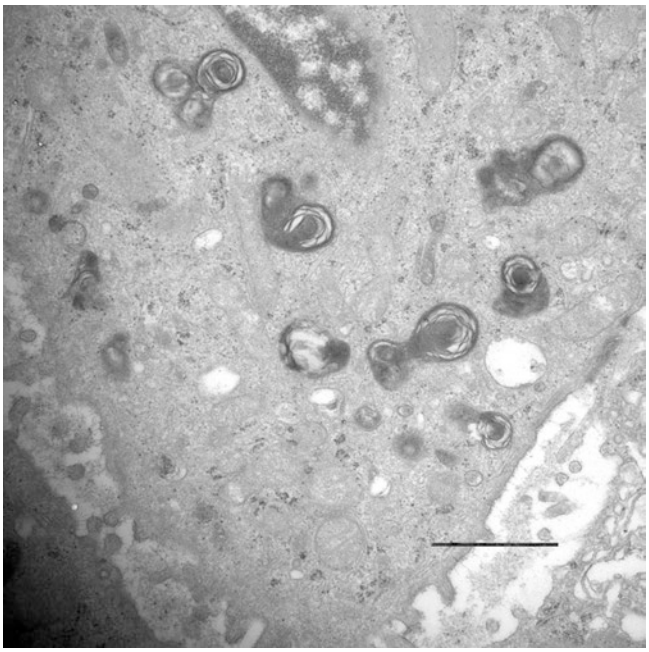

Fig. 8.2 Electron microscopy of adenocarcinoma cells in a pleural effusion. The multilamellated bodies indicate the differentiation into a pneumocyte phenotype. Bar 5 μm

be characterized by showing immunoreactivity to epitopes like CD56, chromogranin, and synaptophysin. Biologically, these tumors differ from other forms of invasive lung cancer, and the main divider in classification of lung cancers is the neuroendocrine SCLC vs. NSCLC.

A number of different terms have previously been used for this biologically separate category of small-cell lung cancers: small-cell anaplastic carcinoma, oat-cell carcinoma, lymphocyte-like carcinoma, and recently also neuroendocrine carcinoma grade 3. To be categorized into this group, the neuroendocrine nature should be established. This can be done by immunocytochemistry (ICC) and/or by electron microscopy. Similar neuroendocrine differentiation is also seen in carcinoids, which can be distinguished based on their rate of proliferation, using the MIB1/Ki-67 antibody. The biology, and perhaps also the presumed histogenesis, of SCLC is, however, quite different from that of the carcinoids, and the term SCLC is preferred for these cancers instead of neuroendocrine cancer grade 3.

LCC constitutes a group of non-small-cell carcinomas that are too poorly differentiated to allow the distinction between epidermoid or glandular differentiation. The proportion of cases referred to this group varies from one material to another, and with the adjunct of ICC, many of these cases will be referred to one of the other three groups, large-cell undifferentiated carcinoma becoming in this way rare. A particular form of LCC displays neuroendocrine differentiation (large-cell neuroendocrine cancers, LCNEC). The neu-

roendocrine phenotype is established by ICC and distinguished from SCLC by the nuclear size and structure.

Tumors considered to develop from other structures than the epithelium lining the airway surfaces are less frequent. Most common of these are the carcinoids and atypical carcinoids. They belong to the group of neuroendocrine tumors (neuroendocrine cancer grades 1 and 2, respectively) with immunophenotypes similar to the SCLCs. Less commonly carcinomas may also develop from submucosal glands. The tumors formed are identical to those seen in salivary glands, i.e., mucoepidermoid carcinoma, adenoid cystic carcinoma, and epithelial-myoepithelial carcinoma.

The importance of classifying lung cancers is to provide guidance in the choice of therapy and prognosis, i.e., to deduce biological information of clinical importance from the morphology. The number of diagnostically relevant groups is then, however, small. As emphasized earlier, the main divider is the distinction of SCLC from NSCLC. These two groups show major differences in aggressiveness, chemosensitivity, and prognosis, while the different subgroups of NSCLC show only limited variability of these parameters.

One must in this context be aware of the poor correlation between light microscopy, electron microscopy, and ICC, when it comes to subclassification of NSCLCs. Thus in the light microscope, a mixed adenosquamous phenotype is considered to be rare [7]. Electron microscopy, however, reveals that a majority of non-small-cell carcinomas simultaneously exhibit both secretory granules and abundant tonofilaments, in fact indicating that adenosquamous differentiation is the most common phenotype [10].

The use of ICC for classifying lung cancers indicates a spectrum more similar to that obtained with electron microscopy and different from that obtained with routine histology. It therefore seems as if light microscopy has a limited ability to classify the tumors according to their biological behavior, this capacity probably being improved by the adjunct of ICC. Still, for practical reasons, therapy is often based on diagnoses from light microscopy. With increased understanding of factors necessary for drug effects and the development of targeted therapies, a classification based on molecular characteristics will be increasingly important, probably replacing much of the histology.

Etiology and Pathogenesis

Carcinogenesis

Exposure to tobacco smoke, radon, asbestos, arsenic, and other forms of air pollution is the main etiological factor connected to lung cancer. Although smoking is the leading cause of lung cancer in about 80–90% of cases, approximately 10% of patients have never smoked [11]. Environmental factors and genetic susceptibility together are thought to contribute to cancer development. These factors are orchestrated, and they trigger oncogene activation, tumor suppressor gene silencing, and widespread loss of heterozygosity.

Among the 55 carcinogens identified in cigarette smoke, 20 are involved in pulmonary carcinogenesis. Of these, polycyclic aromatic hydrocarbons and tobacco-specific N-nitrosamines,especiallynitrosamine4-(methylnitrosamino)-1-1(3-pyridyl)-1-butanone (NNK), seem to play major roles. The carcinogens start a metabolic activation process, leading to formation of DNA adducts. If the DNA adducts escape cellular clearance and repair mechanisms and persist, they lead to permanent DNA damage, which may hit critical oncogenes such as KRAS, MYC, and tumor suppressor genes including p53, p16, pRB, and FHIT; for review see [11].

Differences in the susceptibility to lung cancer among individuals are likely to occur, and genetic polymorphisms have been identified in proteins associated with carcinogen metabolism. Several novel lung cancer susceptibility genes, located on chromosomes 5p15.33, 6p21, and 15q24-25.1, have been identified by large-scale genome-wide association studies [12]. The 15q25 region contains three nicotine acetylcholine receptor (nAChR) genes [13], and their polymorphisms have also been reported to be associated with nicotine dependence. The 6q23-25 and 13q31.3 regions were also identified as being associated with risk for lung cancer, particularly in never-smokers [12].

Chromosomal Aberrations in Lung Cancer

One of the most frequent and early changes in lung cancer pathogenesis relates to chromosome 3. Amplifications are commonly involving the chromosome arm 3q, and allele losses occur at multiple losses of heterozygosity (LOH) sites on chromosome arm 3p [14]. Frequent regions with amplifications (14q13.3, 12q15, 12p12.1, 8q24.21, 7p11.2, and 8q21.13) and deletions (9p21.3, 9p23, 10q23.31) of lung AC specimens have been identified, residing known oncogenes such as MYC, EGFR, KRAS, and tumor suppressor genes such as CDKN2A/CDKN2B [15] and the thyroid transcription factor (TTF-1) located on chromosome 14q13.3 [16, 17].

A well-known cofactor for lung carcinogenesis is asbestos, a mineral fiber that is known to cause chromosomal aberrations. Lung cancers in patients exposed to asbestos often show a number of additional aberrations (2p21-p16.3, 5q35.3, 9q33.3-q34.11, 9q34.13-q34.3, 11p15.5, 14q11.2, and 19p13.1-p13.3) [18–20].

The Role of Microenvironment in the Survival of Metastatic Cancer Cells in Serosal Effusions

Mesothelial cells play a key role in maintaining the homeostasis of the serosal cavities and possess mechanisms that prevent tumor spread and metastasis [21, 22]. Lung cancer cells, however, show a high predilection to metastasize to the pleural space, where they adopt an anchorage-independent growth in effusion, survive, and proliferate despite the unfavorable condition provided by the serous surface. The molecular basis of this predilection is not fully understood but is most likely based on reciprocal tumor-microenvironment interactions [23–27]. Metastatic tumor cells that disseminate to the serosal cavities possess a strong autonomous proliferative drive, and the presence of malignant cells in the pleural space indicates that the malignant cell has overcome the pleural defense mechanisms [28, 29].

One such potential defense mechanism of the mesothelium against invading malignant cells is endostatin, which inhibits angiogenesis and endothelial cell migration, induces cell cycle arrest and apoptosis, and thereby reduces tumor growth [30]. Polyanionic compounds such as glycosaminoglycans [31] and sialomucins [32] present on the mesothelial surface are other factors having a capacity to counteract tumor attachment and growth. Interestingly, a mechanism by which malignant cells present themselves as innocuous to the mesothelial cellular environment is the expression of the hyaluronan-binding proteoglycan CD44 [33], which acts as a receptor for surfaces carrying hyaluronan.

Cells obtained from malignant pleural effusion show aberrant glucose metabolism [34]. Malignant cells also acquire growth advantage by autocrine and paracrine growth stimulation and developing resistance to apoptosis. They actively modulate the microenvironment in the pleural fluid by inducing a pro-angiogenic shift, by secreting growth factors such as vascular endothelial growth factor (VEGF), basic FGF (bFGF), and transforming growth factor beta (TGF-β) [35–37]) and by inducing benign mesothelial cells to release growth factors [38]. In this way metastatic malignant cells contribute to convert the repressive micromilieu of the pleural space to a permissive one, further facilitating tumor growth. Indeed, the level of VEGF from pleural effusions of lung cancer patients is up to 25-fold higher compared to patients with active infectious diseases [39–43]. Platelet-derived growth factor (PDGF) levels are also selectively higher in lung AC, compared to SCLC and nonmalignant pleural effusions [44]. Various cytokines, interleukins, and interferons, including IL-2, IL-4, TNF-α, and INF-γ, are widespread in malignant effusions, and their relative abundance correlates with each other, suggesting cross talk between them [45, 46].

Recent advances in cancer biology point to a role for inflammatory signaling in cancer. Lung cancer patients with malignant pleural effusion seem to have weaker immune defense than those with TB pleurisy, both locally and systemically [47]. Inflammatory markers were significantly expressed in pleural effusions, and values in pleura-invading tumor-associated effusions in lung cancer patients were typically higher than those of other tumors. IL-8 and VEGF correlated negatively with survival, reflecting to some extent also the tumor origin [48].

The Molecular Biology of Lung Cancer

The molecular signature of lung cancer has been subject of extensive research activity, and NSCLCs, particularly adenocarcinomas, are today very well characterized with regard to their molecular changes. At the same time, emerging data show distinctive molecular signatures also for squamous carcinomas and SCLC; these latter two are, however, not yet included in the clinical routine workflow as no targeted therapeutic options are available. In this chapter the most frequent actionable mutations and molecular changes will be described. These changes carry therapeutic consequences and are already integrated in molecular diagnostics and clinical management as a part of personalized cancer medicine approach. Many laboratories have already integrated ICC- and next-generation sequencing (NGS)-based screening approaches in their workflow, following specific algorithms that allow molecular subtyping, treatment prediction, and selection of patients for targeted therapeutic options.

The Molecular Signature of Lung Adenocarcinoma

Both genetic and epigenetic changes are known to be common events in lung cancer. Driver mutations are responsible for both the initiation and maintenance of the malignancy. To date, *EGFR*, *KRAS*, *BRAF*, *PIK3CA*, and *ERBB2* gene mutations, *EML4-ALK*, *ROS-1*, and *RET-1* fusion genes and *MET* amplifications are the most widely recognized alterations involved in both the biology and the clinical management of NSCLC [49, 50].

EGFR Mutation

The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases (RTKs) is deregulated in a subset of NSCLC by activating mutations, increased copy number, or protein overexpression. *EGFR* is mutated in up to 7–10%

of Caucasian and about 32% of East-Asian patients with NSCLC [51]. Approximately 50% of *EGFR*-mutated cases also show increased *EGFR* copy number [52]. *EGFR* overexpression is present in >60% of metastatic NSCLC and it correlates with poor prognosis [53]. Upon ligand binding, homo- or heterodimerization of EGFR leads to autophosphorylation of the intracellular domain and subsequent activation of the Ras/mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways, resulting in increased cell survival, proliferation, invasion, and metastasis. Activating mutations in exons 18–21 of the *EGFR* gene [54] render tumor cells independent of ligand activation of the TK. Deletions in exon 19 and point mutations of L8555R constitute about 90% of all *EGFR*-activating mutations (for review, see [55]).

Patients with malignant pleural effusions related to lung AC have a higher rate of *EGFR* mutations than patients with primary tumors [56, 57], and this constitutes the most frequent molecular change in pulmonary AC presenting with malignant effusion at the time of the first diagnosis [58]. *EGFR* status predicts tumor responsiveness to treatment and clinical outcome [59–62]. *EGFR* gene mutations were found in the tumor tissue from 25% of NSCLC patients and in 23% of plasma samples [62]. Mutations are most frequently present in females, never-smokers, and ACs with bronchioloalveolar features.

KRAS, BRAF, PIK3CA, and *ERBB2* Mutations

Recent studies showed that activating *EGFR*, *KRAS*, *BRAF*, and *ERBB2* mutations exhibit mutually exclusive patterns in lung AC, suggesting that they represent independent ways of oncogenic pathways [52, 63, 64], and they differ in terms of epidemiological, morphological, biological, and clinical aspects. EGFR and ERBB2 are two signaling receptors upstream of the other three. It is therefore sufficient for the stimulation of MAPK and mTOR in tumor cells if only one of them has a mutation that results in autonomous signaling. This is probably the explanation why they so often are mutually exclusive and indicates that this signaling pathway is important for the development of a lung carcinoma. Still another common situation when mTOR is activated is when there is a loss of the phosphatase and tensin homolog (PTEN) activity. This tumor suppressor gene (TSG) negatively regulates the PI3K activity, and mutations and deletions of *PTEN* then result in increased cell proliferation and reduced apoptosis [15, 65–77].

KRAS mutants show often morphological features of mucinous AC and occur preferentially in males, smokers, and Caucasians [78]. Depending on the screening method used, up to 25% of patients are carrying *KRAS* mutations, whereas mutation of *BRAF* and class-1a phosphoinositide-(3,4,5)-kinase (*PIK3CA*) are less frequent and occur only in <5% of lung cancers [49]. Molecular profiling of metastatic NSCLC derived from malignant effusions shows higher frequency of genetic abnormalities, mainly corresponding to *EGFR* and *KRAS* mutations, together occurring in 59% of the cases [79]. In a clinical mutational profiling of 1006 lung cancers by NGS, the well-known V600E *BRAF* mutation accounted, however, for only 24% of all *BRAF* mutations, whereas kinase-impaired mutations affecting codons 466 and 594 were seen in 25%, highlighting the diversity of BRAF mutations in this setting.

Even though most driver mutations are mutually exclusive, accumulating evidence suggest more complex alterations particularly in advanced cases, involving clonality. A large prospective molecular characterization revealed frequent co-occurring targetable mutations of which some showed at least three concurrent alterations [80], often affecting *EGFR* and *PIK3CA*. Moreover, detailed study of variant allele frequencies together with knowledge of previous EGFR-TKI therapy uncovered the presence of coexisting mutations, one being a dominant, the other a sub-clonal population. In the light of this complexity, a comprehensive broad molecular screening will help us better understand the evolution of individual tumors. Based on this it will be possible to tailor our future therapies, considering also simultaneous targeting of different actionable alterations. At the same time this might pose serious future challenge in defining the best choice of therapy among many possible options. Ex vivo sensitivity testing together with molecular characterization might serve as a useful tool in combining different therapeutic options.

EML4-ALK Rearrangements

The fusion of echinoderm microtubule-associated protein-like 4 (*EML4*) and anaplastic lymphoma kinase (*ALK*) results in constitutive tyrosine kinase activity and activation of the downstream MAP kinase pathway. The *EML4-ALK* fusion gene is formed by a small inversion within chromosome 2p, resulting in the fusion of these genes [81]. It occurs in 3–13 % of NSCLC, and apart from rare exceptions, *EML4-ALK* and *EGFR* mutations are mutually exclusive, but patients share many clinical characteristics [82–85].

EML-ALK rearrangements can be detected by ICC, fluorescent in situ hybridization (FISH), and molecular tests comprising RT-PCR or NGS from cytological specimens obtained from malignant pleural effusions. Sensitive antibodies are now available such as the rabbit monoclonal antibody D5F3 (Ventana Medical Systems, Inc., Switzerland),

described to yield 100% sensitivity and specificity [86]. Recent comparative studies revealed that ICC shows reliable results also when compared to break-apart FISH, often considered the gold standard with high sensitivity, specificity, and positive predictive values [87–89]. Thus, ICC is an excellent tool for screening [90], virtually covering all rearrangements. Discrepancies may, however, occur between various analyses, and the FISH analysis can yield both false-positive and false-negative results. ICC-positive but FISH-negative cases most likely correspond to false-negative FISH results and reflect the limited ability of the FISH analysis to cover all different fusion variants.

NGS offers multiplexed analysis comprising the targetable *ALK*, *ROS1*, and *RET*, among others [91]. By NGS, apart from the *EML4-ALK*, previously unreported fusion partners were identified [92].

ROS1 and *RET* Rearrangements

The ROS proto-oncogene is a RTK with structural similarities to ALK. The precise physiological function of this protein is not known, although it has been associated with cell growth and differentiation. In 1–2% of NSCLC, its gene, *ROS1*, may act as a driver following rearrangement with *CD74*, *EZR*, *SLC24A2*, and *FIG* genes [93]. This translocation is mutually exclusive from *EGFR* mutations and *ALK* rearrangements. The *ROS1* translocation, to which targeted therapies now are available, can be demonstrated by FISH using break-apart probes and by ICC demonstrating the overexpressed protein. Detection of ROS1 with the D4D6 monoclonal antibody (Cell Signaling Technology) may yield false-positive results, and only moderate to strong reactivity should be considered staining >50% of tumor cells [94]. Patients with *ROS1* fusions respond initially to crizotinib, similarly to *ALK* rearrangements, but resistance mechanisms are already known that necessitate new therapeutic strategies to overcome treatment failure [95].

RET rearrangements occur in 1–2% of NSCLC, and the *KIF5B-RET* is the most common fusion gene [96], yielding partial response to cabozantinib in a subset of patients (28%) [97].

Other Mutations

Among other oncogenes, MYC and cyclin D1 are amplified or overexpressed in 5–10% of lung cancer cases [98], whereas the anti-apoptotic Bcl-2 is overexpressed in about 25% of cases [99]. These alterations, however, are not targeted in clinical settings.

MET exon 14 skipping mutations and high-level amplification in the *MET* gene also occur relatively to a high extent, ranging from 3 to 17%, respectively, in various types of lung cancer and indicating poor prognosis [100, 101]. They also open up for new therapeutic options and can serve as useful biomarkers [102, 103].

Small-Cell Lung Cancer (SCLC)

The molecular biology of SCLC differs greatly or in many aspects from NSCLC [104].

Dominant oncogenes of the *MYC* family are frequently overexpressed in both SCLC and NSCLC, while the *KRAS* oncogene is never mutated in SCLC but is mutated in 30% of NSCLCs.

The most frequent genetic abnormalities involve TSGs. SCLC and NSCLC differ significantly also in the TSGs that are inactivated during the pathogenesis of lung cancer. There were 22 different "hot spots" for loss of heterozygosity, 13 of them with a preference for SCLC, 7 for NSCLC, and 2 affecting both. Alterations of both p53 and retinoblastoma suppressor protein (pRB) are central for the carcinogenesis of SCLC. The *TP53* gene, coding for the TSG p53, is mutated in more than 90% of SCLCs and more than 50% of NSCLCs, while pRB is inactivated in over 90% of SCLC but only 15% of NSCLCs. Consequently, p16, which regulates pRB, is almost never mutated in SCLC, while this is found in more than 50% of NSCLCs [105].

MicroRNAs in Lung Cancer

MicroRNAs (miRNAs) are small, noncoding, endogenous, single-stranded RNA fragments consisting of approximately 22–23 nucleotides [106, 107]. They play important regulatory roles in a wide variety of developmental and oncogenic pathways [108–112]. Interestingly, genetic dissection of hot spots for chromosomal abnormalities revealed that about half of the miRNAs are located within or near chromosomal fragile sites, common breakpoints, or minimal regions with amplification or loss of heterozygosity [113–115]. The combination of nonrandom chromosomal abnormalities and other genetic alterations or epigenetic events contributes to downregulation or overexpression of miRNAs.

The specific miRNA expression pattern, which characterizes lung cancers, may be useful in the future as a biomarker [116]. A unique miRNA molecular profile, consisting of miR-17-3p, miR-21, miR-106a, miR-146, miR-155, miR-191, miR-192, miR-203, miR-205, miR-210, miR-212, and miR-214, was claimed to be diagnostic of NSCLC [117]. Furthermore, circulating exosomal miRNA signatures mirror those of the primary lung cancer and may discriminate cancer patients from controls.

Detection of miRNA might thus be suitable for screening and early detection of lung cancer [118, 119]. This gives hope also of using them not only as biomarkers but also as therapeutic targets [120].

Gene Expression Profiling

A molecular diagnostic test for distinguishing lung AC from other malignant tumors in pleural effusions has been established [121]. Certain patterns of gene expression have been associated with the different phenotypes of lung cancer and with their prognosis. Thus, deregulation of the Ras oncogenic pathway was found in most lung ACs as opposed to SCCs. Patients with high Ras activity had lower levels of MYC, E2F3, β-catenin, and Src activity, and this pattern could be associated with a less favorable prognosis [122].

Genomic amplification at 3q26.33 has been shown in many cases of lung SCCs. This region contains the transcription factor SOX2, which is necessary for squamous differentiation. Furthermore, SOX2 expression is required for proliferation and anchorage-independent growth of lung cancer cell lines, and SOX2-driven tumors show expression of markers of both squamous differentiation and pluripotency [66].

Activation of the WNT pathway was identified as a determinant of metastasis to the brain and bone during lung AC progression. Data are, however, lacking regarding the involvement of this pathway in metastatic spread to the pleura [123].

Epigenetic Alterations

Epigenetic alterations are considered to play important roles in lung cancer. Hypermethylation of the promoter region of key genes is one of the most common mechanisms that tumors use to inactivate the function of tumor suppressor and other genes. Epigenetic analysis of pleural fluid improves the diagnostic yield and accuracy of the current cytologic examination [124]. Hypermethylation [125] or homozygous deletion of p16 [126] is frequently detected in malignant pleural fluids. Significant differences were also detected in the methylation profiles between the two major types of NSCLC, whereas SCLC clustered together with carcinoids [127]. Patients with methylation of *p16INK4a*, RAS association domain family 1A (*RASSF1A*), or retinoic acid receptor *β* (*RARβ*) were 5.68 times more likely to have malignant effusions than patients without methylation. Furthermore, methylations per patient were more numerous for lung cancer patients than for nonmalignant pulmonary conditions [128]. Differences in the frequency of *RARβ* methylation pattern correspond to 70% for SCLC and 40% for NSCLCs [105].

Interestingly, *KRAS* mutations were significantly higher in *p16* (*INK4A*)-methylated cases than in unmethylated cases, and the methylation index was higher in *KRAS*-mutant cases than in wild-type cases [129].

A comparison of mutation and methylation demonstrated that *EGFR* mutation had an inverse correlation with methylation of *SPARC* (secreted protein acidic and rich in cysteine), an extracellular Ca^{2+}-binding glycoprotein associated with the regulation of cell adhesion and growth, and the *p16INK4A* gene [130].

Integrative Approach to Molecular Profiling

The integrative approach to analyze parallel dimensions enables the identification of genes that are disrupted by multiple mechanisms and/or pathways that are disrupted at multiple components at low frequency. The MUC1 glycoprotein interacts with EGFR, ERBB2, and c-Src in a way that activates cell proliferation. EGFR here seems to regulate the binding of MUC1 to c-Src [131, 132]. The MUC1 gene shows such a concerted disruption, displaying concurrent copy number increase, hypomethylation, and overexpression [133].

Proteomics

Expression patterns obtained with genomic analyses are preferably paralleled with corresponding wide screening for the pattern of proteins formed. The techniques for such analyses develop rapidly, and thousands of proteins can now be identified using a tumor volume of 0.01 mm^3 [99]. Studies have indicated that the protein patterns can be used for establishing the presence of a lung cancer and to further indicate the histological type of tumor [99, 111]. It has also been possible to correlate the obtained protein patterns with prognosis and even to indicate possible therapeutic targets [99, 111–113]. These studies have mainly analyzed proteins obtained from the tumor tissue, but similar results can also be obtained by analyzing effusion supernatants and serum [134–136]. This possibility for a wide proteomics screen is highly promising. The analysis can reveal novel biomarkers and specific expression patterns as a diagnostic tool that extends far beyond the determination of only a few biomarkers. The analyses still await standardization for use in clinical routines. Once this is done, the clinical utility of effusion analyses may increase greatly. Integrative approaches, adding also RNA sequencing to DNA and proteomic data, will improve this molecular characterization of tumors.

Ancillary Methods in Diagnostic Effusion Cytology

One cause of an effusion is the establishment of a malignant condition in the serous cavity. When the fluid is taken for diagnostic examination by clinical cytology, the primary question is always whether there is a malignant condition or not. There are, however, conditions when the mesothelium is stimulated to proliferate for other reasons. This stimulation will change the morphology of the mesothelial cells, which will be polymorphic with distinct and sometimes multiple nucleoli, and the cells will pile up to form papillary structures. This proliferative process, also called "mesotheliosis," is perhaps the most difficult pitfall in effusion cytology. Therefore, a correct malignant diagnosis often requires the help of adjuvant analyses, either ICC [137, 138] or molecular biology techniques [139, 140], as described elsewhere in this book.

The most common primary for a malignant involvement of the pleura is a lung cancer. The tumors usually shed both dissociated cells and cell groups into the fluid. The basic morphology of these cells does not differ significantly in cytological preparations from the primary tumor. Among the NSCLCs, however, the adenomatous differentiation is by far the most common. It may be that peripheral lung carcinomas, more often being ACs, will spread to the serous cavity earlier than centrally growing tumors. This is, however, not the entire explanation. Other factors must also contribute, and reciprocal tumor-microenvironment interactions are most likely to be involved. The diagnostic features for these tumors and a substantial amount of possible ICC adjuncts are described elsewhere in this book. It may be wise to routinely include a minimal battery of these ICC reactions whenever diagnosing a malignant effusion: thyroid transcription factor-1 (TTF-1) to support lung origin, CK5 and p63 to show squamous differentiation, CK7 for adenomatous cells, and in case of small-cell morphology also CD56, synaptophysin, and chromogranin.

Electron Microscopy

Electron microscopy of effusion cell pellets can be an adjunct, although its role in diagnostic effusion cytology is limited. This analysis of an effusion cell pellet is most often employed to establish the diagnosis of a malignant mesothelioma, but it can sometimes also define the phenotype in metastatic lung carcinomas. The ultrastructural presentation of the adenomatous and epidermoid phenotypes is well known, but will classify the tumor cells different from light microscopy, sometimes with tonofilaments and secretory vacuoles simultaneously present in the same cell [10]. In particular, there are two tumor types that can be recognized at the ultrastructural level. The first of these is AC cell with the pneumocyte type 2 phenotype that contains the typical multilaminated bodies associated with the production of surfactant (Fig. 8.2). The second main type of lung cancer that can be recognized by electron microscopy of an effusion cell pellet is the SCLC. Cells of this phenotype contain electron-dense neurosecretory granules, supporting a diagnosis of neuroendocrine cancer. This diagnosis is, however, often better achieved with ICC.

Analysis of Aneuploidy by FISH

Malignant cells in effusions are readily demonstrated with the UroVysion kit (Abbott Molecular Inc., Des Plaines, IL), labeling the 9p21 locus (p16 region) and the centromeric regions of chromosomes 3, 7, and 17 [139]. Similar accurate definition of malignancy can be obtained with a set of probes labeling 5p15.2, 6p11.1-q11, 7p12 (*EGFR*), and 8q24.12–24.13 (*CMYC*) [141]. The probes were formerly offered as a kit ("LaVysion," Abbott Molecular Inc.), particularly aiming for the detection of lung cancer in cytologic specimen, and they are now available as isolated reagents. While these reagents reveal the presence of a malignant condition, there are so far no established and routinely used techniques that provide information regarding tumor origin or tumor type.

NGS

NGS is already incorporated in the clinical workflow of many laboratories. Actionable mutations can be detected by specifically tailored lung cancer-related gene panels comprising a limited number of genes. Regardless of the method used, multiplexed molecular profiling of pleural effusions includes typically *EGFR*, *KRAS*, *BRAF*, *PIK3CA*, *NRAS*, *MEK1*, *AKT1*, *PTEN*, *HER2*, *MET*, *FGFR1*, *FGFR2* and *ALK*, *ROS1*, and *RET* fusions [142]. However, considerable challenges are posed by the bioinformatics, as lung cancer panels are gradually expanded to whole exome sequencing (WES) and whole genome sequencing (WGS) [143]. This analytical challenge might limit the broad clinical applicability of NGS for genotype-tailored treatments [144].

Treatment Options

A malignant effusion corresponds to a disseminated tumor beyond possibilities to cure. Thus, chemotherapy with a palliative purpose or best supportive care is the main therapeutic alternative. Severe dyspnea occurs in 60–80% of the patients

with malignant pleural effusion; therefore its management is primarily aimed to reduce symptoms by repeated pleurocentesis or pleurodesis. Pleurodesis involves insufflation of a sclerosing agent, most often talc, into the pleural space, causing an acute inflammatory response, followed by an extensive fibrosis, thus preventing the recurrence of malignant pleural effusions [145]. Talc insufflation alters the angiogenic balance in the pleural space from a biologically active and angiogenic environment to a more angiostatic milieu [146], and a large surface area covered with normal mesothelial cells is a prerequisite for a successful pleurodesis.

Multiple trials have established the benefit of chemotherapy for palliation and disease control of patients with malignant effusion, compared to best supportive care [147–149]. The response to therapy, however, differs between the tumor phenotypes, the largest difference being between SCLC and NSCLC. For optimal therapy it is therefore important not only to establish the malignant condition but also to obtain a more detailed diagnosis of tumor phenotype.

Chemotherapy Regimens Based on Clinical Trials and Empirical Data

NSCLC

Chemotherapy prolongs the survival of patients with advanced NSCLC when compared to best supportive care alone. Platinum-based combination chemotherapy seems to be the most effective according to meta-analyses [149, 150]. Among the two most frequently used platinum-based drugs, carboplatin has a more favorable toxicity profile and similar efficacy compared to cisplatin, which is highly nephrotoxic [151]. Gemcitabine and paclitaxel are anticancer agents with significant single-agent activity against advanced NSCLC. They have different mechanisms of action and their toxicities are nonoverlapping [152], which also makes them attractive in combination treatment. Indeed, adding carboplatin to either gemcitabine or paclitaxel resulted in better response and survival rates [153, 154]. Drugs that may be combined with platinum include the third-generation cytotoxic drugs docetaxel, gemcitabine, irinotecan, paclitaxel, pemetrexed, and vinorelbine [155].

SCLC

SCLC is considered a chemotherapy-responsive disease, and etoposide-platinum is the standard first-line treatment. Despite initial response rates of more than 60% of the patients and complete response rates of 20–30%, the median survival time and efficacy of systemic chemotherapy have not been significantly improved in the past decades [156]. Taxanes, topoisomerase inhibitors, and antimetabolites such as pemetrexed and gemcitabine have been demonstrated to be efficient both as single drugs and in combination with platinum-based drugs [157, 158].

Targeted Therapy

With the identification of driver mutations in patients with defined clinical and morphological characteristics, a new arsenal of therapeutic options is available for the treatment of patients with lung cancer [159, 160]. A recent prospective study revealed that a high proportion of patients harboring sensitizing *EGFR* mutations or *ALK* and *ROS1* fusions received matched targeted therapy and also showed clinical benefit in most cases [80], highlighting the impact of molecular predictive testing for improved clinical outcome.

Targeting Epidermal Growth Factor Receptor (EGFR)

The most widely studied targeted therapy is related to the epidermal growth factor (EGF) pathway [161]. Patients with advanced NSCLC harboring *EGFR* mutations have a significantly better response rate when treated with RTK inhibitors than patients with wild-type *EGFR*.

EGFR signaling can be disrupted at numerous points. The most common is the blockade of the cell surface receptor by monoclonal antibodies and inhibition of the activity of the tyrosine kinase domain by tyrosine kinase inhibitors. Only a small proportion of patients will have significant response to EGFR inhibitors in unselected patient material, but the presence of activating mutations in the kinase domain of *EGFR* increases the response rate to 75–90% [159, 161–164].

Patients with pleural effusion showing activating *EGFR* mutations have a significantly better response rate to EGFR tyrosine kinase inhibitors compared to patients with wild-type *EGFR*. Their median progression-free survival corresponded to 11.2 vs. 2.7 months, and overall survival was 21.8 vs. 5.8 months, compared to patients with wild-type *EGFR* [62]. Thus, the presence of *EGFR* mutations highly predicts the efficacy of EGFR tyrosine kinase inhibitors (TKIs) also in advanced NSCLC, giving a significant survival advantage.

Most patients will, however, acquire resistance against TKIs. Major resistance mechanisms comprise a secondary threonine-790 to methionine point mutation (T790M) in the *EGFR* gene and amplification of the *MET* proto-oncogene [165]. The T790M mutation causes steric hindrance and impairs the binding of TKIs. Interference on multiple levels with the EGFR signaling pathway or development of irreversible inhibitors of EGFR may help to overcome this problem. Other frequent mechanisms conferring resistance to TKIs comprise *HER2* and *MET* amplifications and *PIK3CA*

mutation [166]. In a recent study, many T790M-negative patients showed activation of *ERBB2*, *MET*, *FGFR1*, and *ALK* or the RAS/MEK/ERK and PI3K/AKT/mTOR pathways [166]. Furthermore, new resistance-related molecular alterations, such as *TET2* mutation and *SOX2* amplification, were detected.

Targeting EML4-ALK and ROS1

Therapies targeted against ALK are currently under development, and they are already included in clinical trials for NSCLC patients harboring the *ALK4-EML* fusion [81]. Crizotinib, an orally administered dual inhibitor of the c-Met and ALK pathways, has recently been evaluated and showed dramatic clinical benefit for patients with advanced NSCLC. Activation of the analogue *ROS1* gene shows similarly positive results following treatment with crizotinib. However, relapse and acquired resistance mechanisms have also been registered [167].

Targeting the PD-1/PD-L1 Axis

A novel approach to treat NSCLC involves interference with processes that makes it possible for tumor cells to evade recognition of immune cells. In particular the inhibition of the PD-1/PD-L1 axis is now an established and successful therapeutic option [168]. The programmed death-1 receptor (PD-1) is present on activated T cells, and when bound to a PD-L1 ligand on the tumor cells, this has an immunosuppressive effect on the T cell. Tumors that express PD-L1 can be identified by ICC. Attempts to block PD-1 or PD-L1 by antibody-based treatment have efficiently improved the response rates for treatment. In addition to PD-L1 expression, high neoantigen and non-synonymous mutational burden, DNA repair pathway defects with microsatellite instability, mismatch-repair deficiency, and presence of activating T cells are all related to treatment efficacy and improved patient survival [169–171].

Targeting Angiogenesis

Inhibition of VEGF impairs angiogenesis and disrupts metastatic tumor spread. Bevacizumab is a monoclonal antibody that binds to VEGF and blocks interaction with its cell surface receptor. Clinical trials have demonstrated that disruption of these signaling pathways can improve survival in advanced lung cancer. The addition of bevacizumab to paclitaxel and carboplatin improves survival compared with chemotherapy alone in patients with previously untreated metastatic non-squamous NSCLC [172].

Other Agents and Experimental Approaches

Folate antimetabolites (pemetrexed), proteasome inhibitors (bortezomib), modified glutathione analogues, and other agents are currently being evaluated in patients with lung cancer [173]. Experimental evidence suggests that bortezomib is able to specifically target and counteract the effusion-inducing phenotype of lung AC [174]. Bortezomib is a proteasome inhibitor, which targets the ubiquitin-proteasome pathway, with subsequent inhibition of the degradation of proteins involved in cell cycle regulation and cancer cell survival [175]. Recent clinical trials further demonstrate the importance of histology in governing individualized treatment, based on both safety and efficacy considerations. For example, bevacizumab and pemetrexed are currently restricted to patients with non-squamous NSCLC. Bevacizumab causes severe pulmonary hemorrhages in patients with squamous cell histology, whereas pemetrexed seems to be more efficient in patients with non-squamous cell morphology [176].

Assay-Directed Chemotherapy

Systematic reviews of chemotherapy sensitivity and resistance assays performed during the last decades reveal higher response rates for patients receiving assay-guided therapy compared to patients treated with empiric chemotherapy [177, 178]. Of particular interest is optimization of ex vivo assay-based methods selecting treatment regimens with the greatest chance of inducing a response in patients with malignant effusions, since the functional status and short median survival of these patients usually do not allow repeated chemotherapy regimens [179, 180]. These assays have only been applied in a few centers and are not yet integrated into general routine oncology. Reasons for this may be due to problems with performing tumor cell-specific measurements and the lack of larger randomized trials. The possibility to personalize treatment also including tests of drugs outside standardized first- and second-line regimens is, however, most challenging.

Molecular Biomarkers for Lung Cancer

Diagnostic Tumor Markers

Tumor tissue that has established a metastatic growth in a serous cavity may shed or secrete various cell components into the fluid. These compounds are delivered either as secretory products or as a consequence of tumor cell decay. The demonstration of such biochemical compounds can have diagnostic importance, particularly if the biomarker is unique to the tumor tissue or is associated with drug sensitivity or prognosis. One marker indicating deterioration of cell integrity is cholesterol, and together with the simultaneous determination of more specific tumor markers such as CEA, it is possible to indicate presence of a malignant condition [181–184]. Attempts to define malignant involvement of the serous cavities by biomarker analyses specifically directed toward malignancy-associated epitopes included also Her-2/neu

[185], CYFRA 21-1 [186, 187], CA-19.9 [188], CA 125 [189, 190] CA15-3, VEGF [191], and HGF/SF [192]. Similarly, the measurement of TTF-1 and napsin A can be used to define the presence of a bronchogenic carcinoma.

Predictive Markers for Optimal Treatment Response

The goal in the management of lung cancer is to achieve optimal treatment response for each patient. However, only a minority of patients benefit from a given cancer treatment. This has led to interest in the identification of gene expression-based predictive signatures. Given the high biological heterogeneity of lung cancer, molecular biomarkers are required for optimal decision-making and to predict the likelihood of success or failure of a given therapy. A well-validated genotyping can give a good basis for personalized treatment.

Prediction of EGFR Tyrosine Kinase Inhibitors

The observation that only a minority of patients responds to EGFR-targeted therapies, in combination with their toxicity and high costs, has driven the search for validating molecular markers which can predict treatment response [193]. Screening for *EGFR* mutation status is to date the most relevant approach for selecting lung cancer patients for treatment [52, 194]. Apart from the malignant cells, the cell-free pleural fluid may also be a feasible clinical specimen for *EGFR* mutation detection in advanced NSCLC, if proper and sensitive detection methods are employed [195]. As direct sequencing can miss a significant portion of mutations in these heterogeneous specimens, more sensitive methods, such as mutant-enriched PCR and gene scan, may provide more reliable mutational information [196–198].

EGFR amplifications are less informative from a clinical point of view, since *EGFR* mutations relate best to treatment response to EGFR tyrosine kinase inhibitors. Patients with tumors lacking *EGFR* mutations and with *EGFR* amplification have dramatically lower response rates, corresponding to approximately 8% [199] compared to 70–90% for those with *EGFR* mutations. In addition to molecular methods, EGFR can be demonstrated by ICC, and antibodies specifically directed toward the mutated *EGFR* epitopes are available. This provides an alternative way to predict response to EGFR inhibitors. This is particularly useful on effusions with insufficient cells for molecular testing [200].

Markers Indicating Primary or Acquired Resistance to EGFR Inhibitors

Primary resistance to EGFR TKIs is seen in association with activating mutations of downstream compounds. Thus lung ACs, harboring activating mutations in the downstream *KRAS*, are associated with a lack of sensitivity to gefitinib (Iressa) and erlotinib (Tarceva), suggesting that treatment decisions regarding use of these kinase inhibitors might be improved by determining the mutational status not only of *EGFR* but also *KRAS* [64], although the two often are mutually exclusive. Activating mutations on codons 12, 13, and 61 of *KRAS* are predictors of resistance to EGFR inhibitors and of poor prognosis. Mutations in the *KRAS* oncogene constitute a negative predictive marker in this clinical setting, and their presence can be used to predict which patients are unlikely to benefit from treatment with EGFR-directed therapy [52, 201]. Similarly, patients with EML4-ALK fusions do not benefit from EGFR tyrosine kinase-based therapy [84].

Acquired resistance to EGFR inhibitors is often connected to amplification of the gene encoding for the MET receptor or a second gatekeeper threonine-790 to methionine point mutation (T790M) [202–204]. Activating mutations of the main downstream effectors of *KRAS*, i.e., *BRAF* (V600E), also signal treatment failure with EGFR inhibitors [205]. Other parameters indicating acquired resistance to EGFR inhibitors are EGFR polysomy, mutations in codons 9 and 20 of the lipid kinase *PIK3CA*, expression of PTEN, which causes the inhibition of *PIK3CA*. Homozygous loss of *PTEN* contributes to erlotinib resistance in *EGFR*-mutant lung cancer by activation of Akt and EGFR [70].

Predictive Markers for Treatment Response

Thymidylate synthase (TS) catalyzes reductive methylation of deoxyuridine monophosphate (dUMP) to deoxythymidinemonophosphate (dTMP), providing the only de novo source of thymidylate required for DNA replication and DNA repair [206]. This enzyme is the primary target of pemetrexed (Alimta), and high expression levels counteract the effects of this drug, making the tumor resistant [206–208].

Advanced NSCLC expresses excision repair cross-complementing group 1 gene (ERCC1) and ribonucleotide reductase subunit M1 (RRM1) in 35% and 40% of patients, respectively. This expression, whether determined by ICC or RT-PCR, predicts resistance to platinum-based drugs and an unfavorable outcome after platinum-based treatment [209–212].

Drugs like vinorelbine, taxane, and paclitaxel are antimitotic agents, with preferential action directed against tubulin. In NSCLCs the expression of IIIβ tubulin is reported to indicate resistance to such microtubule inhibition [213–215].

Predictive Biomarkers in Clinical Trials

Lung cancer clinical trials account for 14% of ongoing oncology trials worldwide [216]. Although biomarker analysis was included in 38% of the ongoing NSCLC clinical trials registered in the ClinicalTrials.gov website, only 8% of the trials used actual biomarkers for patient selection. EGFR expression or mutation status was the most common bio-

marker, used to select patients in 44% of clinical trials, followed by *KRAS* mutation status in 13% of the trials [217]. Molecular tests including *EGFR*, *KRAS*, *ERCC1*, *RRM1*, VEGF, and serum tumor markers are not routinely used yet, but they might have clinical relevance in the near future [155].

Prognostic Biomarkers

Lung AC is one of the most frequent metastatic tumors occurring in the serosal cavities [218, 219]. It often causes a malignant effusion corresponding to a disseminated disease beyond possibilities to cure [220]. Patients with malignant effusion have a limited life expectancy, with median survival times ranging from 4 to13 months in different studies [221, 222]. A number of biomarkers have been suggested to distinguish patients with better prognosis. A meta-analysis based on 53 published studies identified *KRAS* mutation as a negative prognostic factor [223], while *EGFR* mutations were associated with a better prognosis [224]. Gene expression-based prognostic signatures for NSCLC have, however, not yet been standardized for clinical application [225].

Attempts have also been done to find prognostic markers by genome-wide screening and ICC. Using a tissue microarray from NSCLC specimens, it could be shown that syndecan-1 and EGFR expression was associated with a 30% reduction in the risk of death, independent of histology and other confounders. It can be hypothesized that loss of expression of these receptors reflects a less differentiated tumor with a more pronounced biologic aggressiveness, explaining the worse outcome for patients with such tumors [226].

On the other hand many markers detected in pleural fluids are negatively correlated to patient survival such as survivin [227, 228], IL-8, VEGF [48, 229], lactate dehydrogenase [230], and weak telomerase activity [231].

Concluding Remarks

Lung carcinoma cells exfoliated into an effusion can often provide a diagnostic basis sufficient for clinical management. The development of new analytical techniques and the increased understanding of tumors will gradually shift the focus of tumor characterization toward biological parameters defined by molecular biology, epigenetics, and protein expression. This means that the analysis of isolated cells will be increasingly important for the choice of therapy and the diagnostic information can be made available earlier in the diagnostic process.

Tumor cells from an effusion can routinely be obtained without previous aldehyde fixation and will therefore provide a better material for the analysis of their DNA or protein contents, as compared to paraffin embedded tissues. Furthermore, the spread of a lung cancer to a serous cavity implies a more advanced stage of the disease. It can therefore be recommended in these cases that the search for therapy targets preferably should be performed using cells from the effusion rather than from the primary tumor tissue. Such a development toward increased use of cytological material requires attention to the handling of samples, perhaps including the development of routines for tumor cell enrichment and cell culturing.

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