6 Molecular Cytology of Serous Effusions

Ben Davidson

6.1 Introduction

Metastasis is a decisive event in tumor progression and the presence of cancer cells outside the organ of origin dictates in the majority of cases a need to explore treatment modalities beyond surgery. This is particularly true for malignant effusions, since tumor cells within the peritoneal, pleural, and pericardial spaces cannot be surgically removed. Chemotherapy and radiotherapy, while highly effective in many cancers, are usually unable to eliminate all tumor cells.

Among the primary cancers of the serosal cavities are malignant mesothelioma (MM), primary peritoneal carcinoma (PPC), primary effusion lymphoma, and other, rarer entities. The majority of tumors affecting the serosal cavities are nevertheless metastatic, constituting in adults most often adenocarcinomas of the breast, lung, ovary, or gastrointestinal tract. Other carcinomas and hematological cancers, as well as sarcomas, germ cell tumors, and malignant melanomas, are less frequently encountered but have all been described at this anatomic site [1].

Molecular techniques have become central in cancer management in recent years and are used as aids in the diagnostic setting, as well as in assessing therapeutic options, in predicting treatment response, and in prognostication. Effusions are ideal specimens for molecular analysis, as they often contain large numbers of viable cells in suspension, often dissociated or in small groups. Effusion supernatants are also informative, as they contain DNA, RNA, microRNA, or protein from tumor, as well as host cells. Virtually any molecular technique, including high-throughput analyses, has been applied to effusion specimens, and considerable knowledge has

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been gained in these studies [2]. Translation of these studies into clinical practice has, as in many other settings, nevertheless been slower and of more limited scope, and many of these publications represent single studies that have not been reproduced by other investigators. Others, e.g., telomerase assays, have been studied by several groups and yet have failed to become standard practice. This chapter focuses on diagnostic and therapy-related tests which constitute current practice, at least in tertiary cancer centers. The use of these tests is dictated by the origin of the tumor diagnosed in the effusion specimen and thereby does not represent an assay specific for cancers at this anatomic site. Rather, effusions are one of several types of specimens, including fine-needle aspirates and biopsies, which may be studied using the same technology. Hematological cancers are not discussed in this chapter.

6.2 Molecular Tests Applied to Effusion Diagnosis

The two main molecular assays applied to effusion diagnosis are in situ hybridization (ISH) and polymerase chain reaction (PCR).

ISH is a commonly used method which has the advantage of combining molecular analysis with morphological assessment. Visualization may be achieved using a colorimetric assay (chromogenic ISH, CISH), silver staining (SISH), or fluorescence (FISH). Within the diagnostic context, FISH is the most frequently applied test.

Han et al. analyzed 72 malignant effusions from patients with different cancers, of which the majority were lung carcinomas, and 21 benign effusions using probes for chromosomes 7, 11, and 17. The observed sensitivity and specificity combining morphology and FISH were 88% and 94.5%, respectively [3]. Rosolen and co-workers studied 200 effusions, including 82 cytologically malignant specimens, 67 suspicious ones, and 51 cases diagnosed as benign, applying FISH probes for chromosomes 7 and 17. FISH confirmed the cytological diagnosis in malignant and benign specimens and aided in detecting malignant cells in cases with inconclusive cytology [4]. FISH analysis using probes for chromosomes 11 and 17 was found to be useful in differentiating malignant from benign effusions in another series [5].

FISH has been used as a tool for diagnosing MM in several studies, applying probes detecting chromosomal aberrations which frequently occur in this cancer, in particular the homozygous deletion of the *CDKN2A* gene, encoding the tumor suppressor proteins p14 and p16 at chromosome 9p21. Deletion at this chromosomal site was shown to be a common event in MM and effectively differentiated this tumor from benign effusions in three studies [6–8]. The presence of homozygous *CDKN2A* deletion was shown to be closely similar in effusion specimens and patient-matched biopsies in two recent studies, of which one showed the same agreement for BAP1 immunohistochemistry (IHC) [9, 10]. Combination of *CDKN2A* by FISH and BAP1 by IHC was reported to be useful in a recent study of 67 effusions (32 MM, 35 atypical mesothelial proliferations), of which 38 were analyzed using both methods [11].

The UroVysion™ kit, containing centromeric probes for chromosomes 3, 7, and 17 and a probe for chromosome 9p21, has been applied to effusion diagnosis, with focus on MM.

Analysis of 68 effusions, including 21 MM, 29 metastatic tumors, mainly of lung and breast origin, and 18 reactive specimens, showed 9p21 deletions in 12/21 MM and 3/29 metastases and none of the reactive specimens. Gains at 9p21 were more common in metastases, while gains in chromosomes 3, 7, and 17 were frequent in both MM and metastases [12].

In another study, in which 52 MM and 28 reactive effusions were analyzed, positive FISH analysis, most frequently 9p21 deletion, was found in 41/52 (79%) MM compared to 0/28 reactive specimens [13]. FISH analysis using centromeric probes for chromosomes 7 and 9 was found to be useful in differentiating MM from benign effusions in another study [14].

Example of the 9p21 FISH assay is shown in Fig. 6.1.

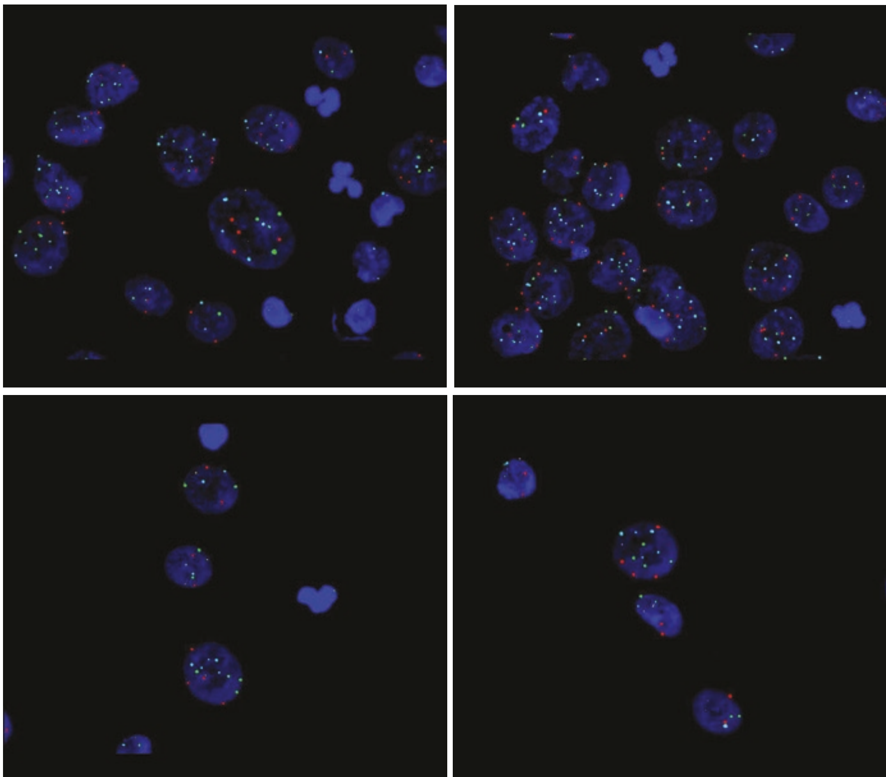


Fig. 6.1 Chromosome 9p21 deletion by fluorescent in situ hybridization (FISH). Malignant mesothelioma (MM) pleural effusion analyzed using a probe for chromosome 9p21 (UroVysion™ kit). MM cells lack yellow dots, corresponding to homozygous chromosome 9p21 deletion. (Courtesy Prof. Anders Hjerpe, Karolinska Institute, Stockholm, Sweden)

Several other groups have reported on ISH- or PCR-based assays as adjuncts to morphology in effusion diagnosis. However, these have been single reports which are yet to be validated. In two studies using ISH, [35S]UTP-labeled probes against *MUC2* and *MUC5AC* were applied to pseudomyxoma peritonei specimens [15], and thyroid transcription factor-1 (*TTF1*) gene amplification by FISH was analyzed in lung carcinoma [16].

Quantitative RT-PCR (qRT-PCR) assay analyzing the expression of the mucin genes *MUC1*, *MUC2*, and *MUC5AC* in 112 pleural effusions found *MUC1* and *MUC5AC* to be sensitive and specific in the diagnosis of malignancy [17]. Similar results were reported for an RT-PCR assay detecting *EGP2* (*EPCAM*) [18] and for the melanoma-associated antigen (MAGE) family members *MAGE1* and *MAGE3* and the related genes *BAGE* and *GAGE1-2* [19]. An RT-PCR assay for prepro-gastrin-releasing peptide (prepro-GRP) detected small cell lung carcinoma in effusion specimens [20], while an assay detecting the mammaglobin and mammaglobin B genes *hMAM* and *hMAMB* was positive in effusions from patients with breast carcinoma, as well as other gynecologic carcinomas and lung carcinoma [21]. The combined use of *CLDN4*, *EPCAM*, and *CK20* PCR was suggested as adjunct to cytology in another study [22].

Analysis of effusion supernatants for cyclin E gene copy number by qPCR [23] or *BIRC5* mRNA levels [24] was similarly reported to effectively differentiate malignant from benign effusions.

6.3 Molecular Tests Applied to Effusions as Predictive Test

ISH and PCR have in recent years been applied to evaluate the presence and expression level of molecules which may be targeted in different cancers, particularly HER2 and epidermal growth factor receptor (EGFR) and related molecules.

6.3.1 HER2 Status

HER2 amplification is present in 20–25% of breast carcinomas and is associated with aggressive disease. HER2 is targeted by the monoclonal antibodies trastuzumab (Herceptin®) and pertuzumab (PERJETA™) and by the tyrosine kinase inhibitors (TKIs) lapatinib (Tykerb®), afatinib, and neratinib (HKI-272) [25]. Trastuzumab is additionally used in treating gastroesophageal carcinomas that overexpress HER2 [26], as well as in a subgroup of patients with HER2-overexpressing colorectal carcinoma [27]. HER2 status is evaluated at the protein level using IHC or at the gene level using CISH, SISH, or FISH (Figs. 6.2 and 6.3).

A comprehensive review of 47 studies in which 3384 patient-matched primary breast carcinomas and metastases were compared, with focus on solid lesions, showed that HER2, as well as hormone receptor expression, is not infrequently discordant between primary and metastatic breast carcinoma, highlighting the

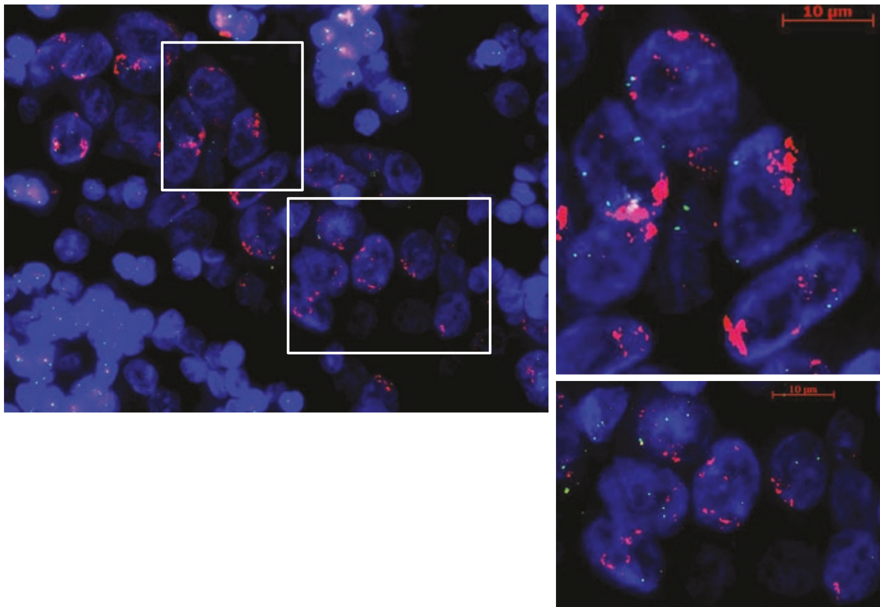
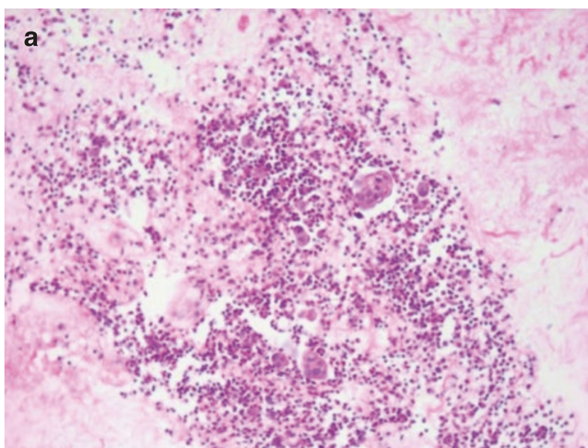


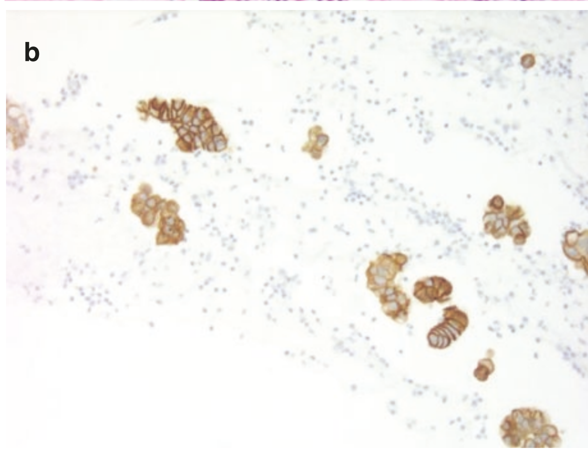
Fig. 6.2 HER2 amplification by FISH. Left: two clusters of tumor cells (marked by frame), in which cells have HER2 copy number >20 (pink clusters), while the CEP17 centrosomal probe detects 1–2 copies (green dots) per cell, evidence of HER2 amplification. High-power detail is shown to the right. (Courtesy Dr. Hege Russnes, Oslo University Hospital)

relevance of testing metastases for HER2 status [28]. Several studies have focused on effusion specimens in this context.

Shabaik et al. compared HER2 status by IHC in cell blocks from cytological specimens (n = 42), including 15 effusions, and 40 patient-matched core biopsies, and found good agreement between these specimens, suggesting that cell blocks constitute relevant specimens for this analysis. Additionally, results using IHC and FISH, the latter performed in seven cases, correlated well [29]. HER2 status by IHC and FISH correlated less well in another study of 35 effusions (31 breast and 4 ovarian carcinomas), in part due to chromosome 17 polyploidy [30]. Arihiro et al. compared HER2 status by FISH in 100 pairs of primary breast carcinoma and locoregional recurrences or metastases, including 7 effusions, and found discrepancy in 9 cases, including both negative-to-positive and positive-to-negative conversions [31]. In a recent, smaller study, concordance in HER2 status was seen in eight pleural effusions compared to the primary breast carcinoma, whereas one ascites specimen showed positive-to-negative conversion [32].

Data for gastric carcinoma is more limited. However, analysis of 72 patient-matched primary and metastatic gastric carcinomas, including 15 effusions, showed high concordance rates for HER2 status by both FISH (98.5%) and IHC (94.9%) [33].





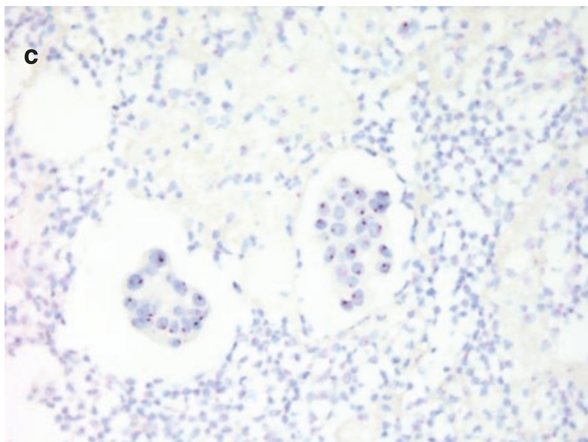


Fig. 6.3 HER2 amplification by silver in situ hybridization (SISH). (**a**) H&E-stained section from a breast carcinoma pleural effusion (×200 magnification); (**b**) HER2 immunostaining, score = 3 (×200 magnification); (**c**) HER2 SISH analysis. Tumor cells have aggregates of black dots, corresponding to HER2 amplification. Chromosome 17 copy number (red dots) does not exceed 2/cell (×400 magnification)

6.3.2 EGFR and Related Molecules

Analysis of *EGFR* mutation status is mandatory prior to TKI treatment and is currently performed in the presence of advanced disease in several cancers, of which the most relevant in the context of effusion cytology is non-small cell lung carcinoma (NSCLC) (Fig. 6.4). *EGFR* mutations are found in 15–20% of lung adenocarcinomas and are limited to exons 18–24, the majority located in exons 18–21. Exon 19 mutations, mainly in-frame deletions, and L858R substitution at exon 21 constitute 85–90% of *EGFR* mutations. The TKIs erlotinib (Tarceva®), gefitinib (Iressa®), and afatinib are approved to the treatment of patients with advanced or recurrent lung cancer which have sensitizing *EGFR* mutations [34].

Testing for *EGFR* mutations can be done using different methods, including direct sequencing, denaturing high-performance liquid chromatography (dHPLC),

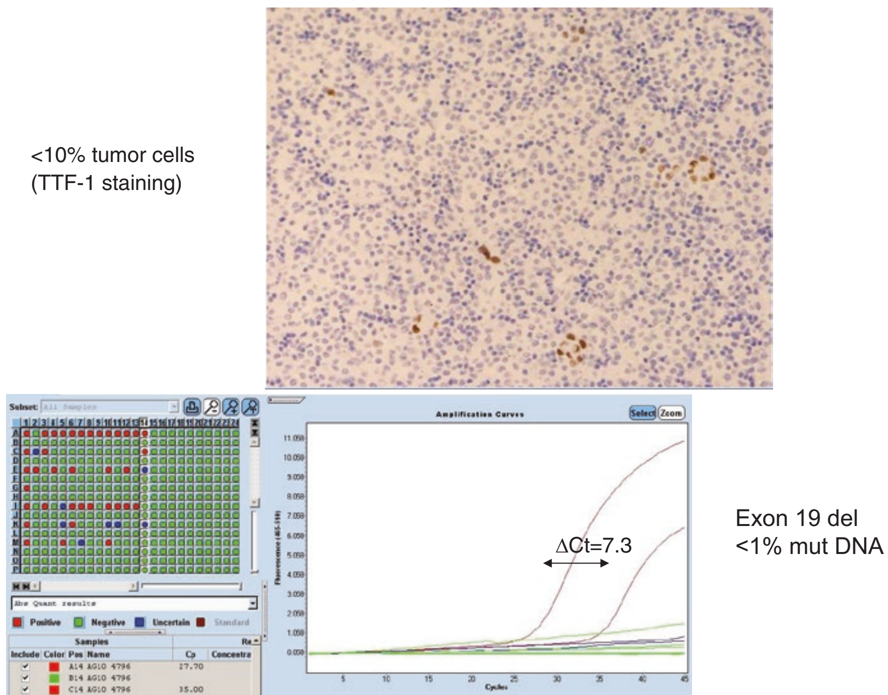


Fig. 6.4 EGFR mutation analysis. Pleural effusion from a NSCLC patient. TTF1 immunostaining shows nuclear expression in tumor cells, which constitute <10% of the cell population. EGFR exon 19 del mutation was nevertheless detected using the therascreen EGFR PCR kit (Qiagen, Manchester, UK) on LightCycler 480 (Roche, Basel, Switzerland). The sample was previously negative using dHPLC, which has lower sensitivity. Mutation was later confirmed in a needle biopsy containing more tumor material (Courtesy Dr. Lilach Kleinberg, Oslo University Hospital)

high-resolution melting analysis (HRMA), pyrosequencing, amplification-refractory mutation system (ARMS) PCR, and PCR-restriction fragment length polymorphisms (PCR-RFLP) [35]. The majority of laboratories use multiplex qPCR-based platforms, such as Cobas (Roche) and Therascreen (Qiagen). Next-generation sequencing (NGS) is likely to play an increasing role in this area in the future [34].

Cytological specimens, including effusions, are considered adequate material for testing *EGFR* mutation status [34, 35], and a growing number of studies have focused on this area in recent years. Success rate was 100% for 5 different methods applied to *EGFR* mutation status analysis in 20 pleural effusions [36]. A concordance rate of 91.7% for histology and cytology was shown in analysis of specimens from 60 patients, in which cytology specimens included 16 pleural effusions and one ascites specimen [37]. Tissue sections, cell blocks, pleural effusions, and sera were studied for *EGFR* mutation status in another study of 37 NSCLC with malignant pleural effusion, in which peptide nucleic acid (PNA)-mediated real-time PCR clamping and direct sequencing were compared. Analysis of the pleural fluid was associated with sensitivity and specificity of 89% and 100%, respectively, compared to tumor tissue and cell blocks using PNA clamping, and 67% and 90%, respectively, using direct sequencing [38].

Comparable values were seen for *KRAS* mutation analysis in another study by the same group, in which 57 malignant effusions, the majority of lung origin, were analyzed using these two methods [39].

In analysis of 48 cytological specimens, including 15 pleural effusions, from patients whose tumors had *EGFR* mutation in tissue specimens, NGS was superior to direct sequencing in detecting *EGFR* mutations (81% vs. 16%, respectively), and mutations were found also in some of the effusions diagnosed as negative for carcinoma based on morphology [40].

Anaplastic lymphoma kinase (ALK) is a protein involved in fetal development, which is lost in adult tissues with the exception of the brain. ALK is expressed in several tumors, including NSCLC, due to genetic rearrangements, most often thorough inversion of chromosome 2p, where the *ALK* gene is located, leading to fusion with the echinoderm microtubule-associated protein-like 4 gene *EML4*, located on the same chromosome arm. The EML4-ALK fusion protein is localized in the cytoplasm following loss of its transmembrane domain, but retains its kinase activity, resulting in pro-survival signaling. *ALK* rearrangements are found in 2–8% of lung carcinomas, and this patient group is eligible for treatment using ALK inhibitors, including crizotinib and newer ALK inhibitors such as ceritinib and alectinib, as well as other drugs currently in development [41].

Soda et al. analyzed 808 lung carcinoma specimens from 754 patients using multiplex PCR and found *EML4-ALK* transcripts in 36 specimens, including 5 pleural effusions, from 32 patients [42]. Wu and co-workers studied pleural effusions from 116 patients with wild-type *EGFR*. *EML4-ALK* fusion was detected in 39 tumors (34%) using RT-PCR. FISH analysis was positive in 10/12 PCR-positive cases in which a paraffin block from biopsy or surgical resection was available [43]. In another study, *EML4-ALK* fusion was detected in 5/46 pleural effusions with

wild-type *EGFR* using multiplex PCR, whereas 67 specimens with *EGFR* mutation were negative [44].

Other genomic aberrations described in NSCLC affect the *RET*, *ROS1*, *NRG1*, *MET*, *BRAF*, *HER2*, *NF1*, and *MEK1* genes. *RET* rearrangements at chromosome 10 lead to fusion with the *KIF5B* gene, and patients with *RET* rearrangements are currently under consideration for TKI treatment [45]. Analysis of *RET* rearrangements in a series of 722 pleural effusions from patients with lung adenocarcinoma was positive in 17 (2.4), of which 11 and 6 had *KIF5B- RET* and *CCDC6-RET* fusion, respectively [46].

Akamatsu et al. analyzed 100 pleural effusion specimens from 84 patients for *EGFR*, *KRAS*, *BRAF*, *PIK3CA*, *NRAS*, *MEK1*, *AKT1*, *PTEN*, and *HER2* mutations; *EGFR*, *MET*, *FGFR1*, *FGFR2*, and *PIK3CA* amplifications; and *ALK*, *ROS1*, and *RET* fusion genes. *EGFR* mutation was found in specimens from 24 patients, *EML4-ALK* rearrangement in 4 patients, *KRAS* mutation and *EGFR* amplification in 3 patients, and *PIK3CA* mutation and *MET* amplification in 2 patients. *BRAF* mutation, *NRAS* mutation, *AKT* mutation, *ROS1* fusion, and *FGFR1* amplification, the latter reflecting *KIF5B-RET* fusion, were found in one patient each [47].

6.4 Future Directions

While the number of molecular assays that are currently performed on effusion specimens as part of the routine practice of pathology labs is still relatively limited, this is likely to change dramatically over the coming years, as already exemplified by the increasing complexity of lung carcinoma management. While it is fairly certain to assume that FISH and PCR will continue to be an integral part of molecular testing, NGS is expected to have an increasingly central role in this area. Analyzing tumors for genetic changes that are characteristic of each tumor with focus on few dozens of genes is the more relevant assay for assessing patient-tailored therapy, whereas more large-scale platforms are used as discovery tools.

Several recent publications on lung carcinoma are examples of the potential of NGS in this respect. Roscilli and co-workers recently performed mutation analysis of 22 genes in short-term cultures from 16 lung adenocarcinoma effusions and identified mutations in *EGFR*, *KRAS*, *BRAF*, *PIK3CA*, *MET*, *TP53*, and *STK11*, with high variation across tumors. Whole-exome sequencing was performed in five cases and detected multiple mutations affecting critical cellular pathways, particularly in chromosomes 1, 11, and 19 [48]. Analysis of 38 NSCLC pleural effusions using the TruSight™ tumor sequencing panel, which interrogates mutational hotspots in 174 amplicons of 26 genes, identified mutations in *EGFR*, *KRAS*, *BRAF*, *PIK3CA*, *MAPK21*, *PTEN*, and *SMAD4* [49]. DiBardino et al. analyzed 49 NSCLC specimens, including both biopsies and cytological specimens, of which 36 were found to be adequate for full sequencing of 255 genes, including 6/6 pleural effusion specimens, highlighting the value of the latter for such analysis. Using the Illumina HiSeq2500 platform, 179 alterations were found, of which 63 were clinically relevant, including *EGFR*, *KRAS*, *ERBB2*, and *PIK3CA* mutations [50]. The adequacy

of cytological material for NGS was also shown in analysis of 17 specimens, including 4 effusions, tested for alterations in 47 genes, in which mutations in *EGFR*, *KRAS*, *BRAF*, *NRAS*, and *TP53* were found [51].

Studies of other cancers have to date focused on large-scale analyses aimed at mapping the genetic landscape of these tumors.

Lim and co-workers compared normal gastric mucosa, primary carcinoma, and malignant effusions from eight patients and identified mutations characteristic of tumor cells in effusions, which may promote the metastatic process in this cancer [52].

Three studies applied NGS to analysis of ovarian carcinoma effusions. Castellarin and co-workers applied whole-exome sequencing to analysis of serial effusions from three high-grade serous carcinoma (HGSC) patients, including the primary diagnosis specimen, first recurrence, and second recurrence. *TP53* mutations were found in all specimens, and the mutation spectrum of the primary specimen was generally conserved in the subsequent ones, suggesting that chemoresistant clones that are present in the tumor at diagnosis are the origin for recurrent disease [53]. Shah et al. compared the effusion specimen, frozen tumor, and formalin-fixed paraffin-embedded tumor from 5 patients using the IMPACT assay which targets 281 genes. Among 17 mutations found, 10 were detected in both biopsy specimens and effusions and were listed in the Cancer Genome Atlas (TCGA) study, whereas the remaining 7 were detected only in the IMPACT assay. Among the latter, two mutations (in *FGFR3* and *MYB*) were detected only in the effusion specimen from one of the patients [54]. Reinartz et al. analyzed separately tumor-associated macrophages and tumor cells from 28 HGSC and 1 serous borderline tumor effusions using the Illumina HiSeq1500 platform and characterized expression profiles and signaling pathways for each of these cell populations [55].

Effusions are specimens that are relatively easy to obtain, and often contain numerous viable cells, making them ideal for molecular analyses. The studies discussed in this chapter suggest a central role for effusions in cancer diagnosis, as well as tailoring of targeted therapy in the future.

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