Journal of the American Society of Cytopathology (2019) 8, 352–361

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.jascyto.org/

REVIEW ARTICLE

Proceedings of the American Society of Cytopathology companion session at the 2019 United States and Canadian Academy of Pathology Annual meeting, part 2: effusion cytology with focus on theranostics and diagnosis of malignant mesothelioma

Momin T. Siddiqui, MD[a,*], Fernando Schmitt, MD, PhD, FIAC[b], Andrew Churg, MD[c]

[a] *Department of Pathology and Laboratory Medicine, Weill Cornell Medicine, New York, New York*
[b] *Instituto de Patologia e Imunologia Molecular da Universidade do Porto, Porto, Portugal*
[c] *Department of Pathology, Vancouver General Hospital and the University of British Columbia, Vancouver, British Columbia, Canada*

Received 19 June 2019; received in revised form 20 July 2019; accepted 29 July 2019

KEYWORDS
Effusions;
Cytology;
Theranostics;
Mesothelioma;
Serous fluids;
Immunohistochemistry

We live in the "era" of minimally invasive procedures, molecular testing, and personalized care. Effusions have a high sensitivity and will often yield diagnostic cytological material. The companion session presented by the American Society of Cytopathology at the 2019 United States and Canadian Academy of Pathology meeting outlined our current and future projected practices in characterizing, managing, and diagnosing serous cavity fluids. In this second part, the role of theranostics and the diagnosis of malignant mesothelioma, as was discussed at the meeting, have been highlighted. In theranostics, a vast amount of data has been reported regarding the epidermal growth factor receptor and related molecules. Some studies have also reported on HER2 immunohistochemistry and fluorescence in situ hybridization. This follows the most active areas of research in targeted therapy. Furthermore, during this session, malignant mesothelioma was extensively discussed. The cytologic diagnosis of malignant mesothelioma in effusion specimens has been controversial; however, a definitive diagnosis will be possible in many cases. Radiologic information should be sought, because the radiologist can often provide a definite or very likely diagnosis of malignancy.

*Corresponding author: Momin T. Siddiqui, MD; Department of Pathology and Laboratory Medicine, Weill Cornell Medicine, New York Presbyterian Hospital, 525 East 68th Street, F-766A, New York, NY, 10065; Tel.: (212) 746-9347; Fax: (212) 746-8359.
E-mail address: mos9084@med.cornell.edu (M.T. Siddiqui).

https://doi.org/10.1016/j.jasc.2019.07.005

Microscopically, high cellularity and/or numerous balls of cells or papillary groups will favor the diagnosis of mesothelioma. It is important to exclude metastatic carcinoma with a broad-spectrum carcinoma marker, of which claudin-4 has been the best, because it will not cross react with mesothelioma. BAP1 and MTAP immunohistochemistry and *CDKN2A* fluorescence in situ hybridization are very useful adjunctive techniques for separating benign from malignant mesothelial proliferations. The use of 2 of these approaches together will produce a sensitivity of 80% to 90% for epithelial mesotheliomas in the pleura, although the sensitivity has been lower in the peritoneal cavity.

Contents

Introduction

Serous cavity fluids derived from the mesothelium-lined pleural, pericardial, and peritoneal cavities are one of the most common specimen types reported by a cytopathologist. The specimens are relatively simple to procure; however, the diagnosis will be often rendered difficult owing to quantitative and qualitative factors.[1] Identifying only a few diagnostically significant cells in large volumes of fluid can prove difficult to diagnose for a variety of reasons. The presence of abundant inflammation or blood in these specimens will also compound the problem. Furthermore, the morphologic appearance of the cells can lead to interpretive dilemmas that extend beyond morphology into the realm of immunocytochemistry (IHC) and molecular diagnostics.[2-4] IHC can be extremely useful when determining the primary site of the malignancy. However, in many cases, the clinical history and radiological findings will render ancillary testing unnecessary. The high clinical stakes involved in the diagnosis of malignancy in these fluids also adds to the challenge.[5-7] Positive serous cavity fluids often signal end-stage disease progression and will preclude some forms of treatment. However, this could also present opportunities for oncologists to use newer treatment modalities.[8-10] Thus, cytopathologists have been cautious in their management of difficult cases to avoid false-positive or false-negative interpretations that could have a profound effect on the patient's clinical treatment.[11,12] The companion session presented by the American Society of Cytopathology at the 2019 United States and Canadian Academy of Pathology meeting outlined our current and future projected practices in characterizing, managing, and diagnosing serous cavity fluids. In this second part, the role of theranostics and the diagnosis of malignant mesothelioma, as was discussed at the meeting, have been highlighted.

Theranostics

Theranostics is the use of diagnostic tests to select targeted therapy. This is a demanding, emerging area of medicine, especially for practicing pathologists. Although, previously, small tissue biopsy specimens obtained in the context of cancer diagnosis were needed only for diagnostic purposes, they must now also provide enough tissue for molecular testing. This requires not only the presence of tumor cells in the sample, but also their presence in a sufficient amount and percentage of the total cellularity.[13] Serous effusion cytology is able to provide such samples and is considered by most to be equivalent to the use of tissue specimens regarding its adequacy for molecular testing.[6-10] The College of Molecular Pathologists has incorporated these findings in their guidelines for molecular testing for adenocarcinoma of the lung.[14] Many studies have been reported regarding the use of theranostics in serous effusions. Mimicking histologic testing, most studies have focused on

adenocarcinoma of the lung, carcinoma of the breast and ovary, and gastroesophageal adenocarcinoma.

Epidermal growth factor receptor and related molecules

The epidermal growth factor receptor (EGFR) is a gene located on the short arm of chromosome 7, which encodes transmembrane tyrosine kinase receptor of the EGFR (EGFR/HER/ERBB) family.[15] It has also been called HER1 or ERBB1. These receptors participate in intercellular signaling through signal transduction. When a receptor is activated by an external ligand, it dimerizes with another member of the family and then phosphorylates or dephosphorylates certain membrane and cytoplasmic proteins in a cascade that culminates in the transcription and silencing of several genes.[16] This, in turn, will promote cell proliferation, survival, motility, and adhesion, making the EGFR an appealing therapeutic target.

More than 30 drugs have been approved by the Food and Drug Administration (FDA) to target the EGFR. Two are monoclonal antibodies (cetuximab and panitumumab), approved for use in colorectal cancer. The rest are small molecule tyrosine kinase inhibitors (TKIs) and have been categorized as first, second, and third generation. First-generation (eg, erlotinib, gefitinib) and second-generation (eg, dacomitinib, canertinib, afatinib, neratinib) TKIs are competitive inhibitors of EGFR, without selective affinity for non−wild-type (WT) proteins. Thus, they cause significant dose-dependent toxicity related to WT EGFR inhibition. Furthermore, they are susceptible to the development of resistance through mutations in the TK domain (T790M). In contrast, third-generation TKIs (eg, osimertinib, rociletinib, olmutinib, avitinib) have shown nonselectivity for WT EGFR and will form irreversible bonds with mutated EGFR protein moieties. They have activity against EGFR T790M but are also susceptible to the development of resistance through C797S mutation. These molecules have been approved for the treatment of non−small-cell lung cancer (NSCLC).[17] In current clinical practice, the study of EGFR mutation status is mandatory before initiating therapy with the monoclonal antibodies and TKIs. TKIs have been considered first-line therapy for stage IV NSCLC in the current guidelines from the American Society of Clinical Oncology.[18] EGFR mutations will be found in 15% to 20% of lung adenocarcinoma cases and $\leq$5% of lung squamous cell carcinoma cases. They are activating mutations, occurring in predicable locations between exons 18 and 24.[19,20]

Many different methods can be used in the analysis of EFGR mutation status. Direct sequencing, peptide nucleic acid clamping, denaturing high-performance liquid chromatography, pyrosequencing, high-resolution melting analysis, amplification−refractory mutation system polymerase chain reaction (PCR), PCR−restriction fragment length polymorphisms, and commercial quantitative PCR-based platforms, such as Cobas (Roche) and Therascreen (Qiagen).[19,21] Next-generation sequencing (NGS) can also be used to determine the mutation status of the EGFR. The throughput of these systems has been increasing in the past few years, and their cost has been decreasing at a similar rate.[22,23] NGS enables analysis, not only of EGFR mutations, but also of many other genes, even whole exomes or genomes, using only 1 sample.[24] In the lung, this is has been even more relevant, because the current guidelines have recommended that, not only EGFR, but also anaplastic lymphoma kinase (ALK), ROS1, and BRAF, should be tested in all cases of advanced lung adenocarcinoma, irrespective of the clinical characteristics. Even larger gene panels have been used by many institutions.[25]

Testing of EGFR mutation status in cytology specimens has been thoroughly validated, including effusions, for various methods. One study used direct sequencing of exons 18, 19, 20, and 21 in a series of 50 patients with advanced lung adenocarcinoma.[26] They compared the findings from tissue biopsy specimens and pleural effusion cytological material.[26] They did not find a statistically significant difference between sample types in the detection of EGFR mutations.[26] Another group, targeting the same exons with pyrosequencing, found a concordance rate between the histologic and cytologic material of 91.7%.[27] One study used peptide nucleic acid clamping to sequence EGFR on cell blocks and performed a correlation of mutational status with the response to TKI therapy.[28] They found it was highly predictive of the TKI therapy response.[28] However, they did not compare the results with concurrent tissue biopsies.[28] Another study compared peptide nucleic acid clamping with direct sequencing in tissue samples, cell blocks, pleural effusions, and serum.[29] Their results showed greater sensitivity and specificity for clamping across the sample types. They demonstrated sensitivity and specificity similar to those for tissue from both cell blocks and smears. Serum samples had a lower diagnostic value compared with all other samples in their study.[29] In a small series of 36 pleural effusions and 22 matched biopsy specimens, the investigators compared high-resolution melting analysis with direct sequencing.[30] Using cytologic samples, they extracted DNA from both supernatant and cell pellets. They found both sequencing methods to be equivalent in tissue samples; however, the findings from high-resolution melting analysis were superior in cytology.[30] They found that the supernatant had greater DNA content compared with the cell pellets and the sample showed greater sensitivity and specificity values compared with those of the cell pellets, making an argument for the use of cell-free DNA (cfDNA) in serous effusion specimens.[30] These results were replicated in another small series of 32 patients, which found that effusion cfDNA was superior to cell blocks and smears for the genotyping of EGFR, including the detection of the resistance mutation T790M.[31] Two studies used an amplification−refractory mutation system PCR, showing comparable results among cytology, cell blocks, and matched tissue biopsy specimens in >91% of all cases. One of these studies suggested that the results from smear specimens were better than those from cell blocks.[32,33]

A large series was reported in 2017 with >10,000 samples included and 8000 with histologic follow-up data. That series was continued from the findings reported by a previous study. The study was large and focused on sharing an extensive 14-year experience with peritoneal cytologic samples processed using liquid-based cytology.[34,35] They analyzed the role of ancillary techniques as an additional tool in peritoneal samples. Mutational analysis for EGFR and KRAS using the Therascreen (Qiagen) commercial implementation of quantitative PCR was performed. Comparing the findings from cytology and those from matched tissue biopsy specimens, they found a sensitivity of 97.4%, specificity of 98%, diagnostic accuracy of 98%, negative predictive value of 99.7%, and positive predictive value of 99.7% for cytology samples, not only in tumors from the lung, but also in tumors from the gastrointestinal tract.[34,35] Finally, 1 group studied the use of NGS for EGFR mutational analysis in a small series of 48 cases with matched tissue.[36] They compared it with direct sequencing and included both serous effusion specimens and bronchoalveolar lavage specimens. Tumor cellularity was lower than 10% in three quarters of cases. In their series of mostly inadequate material, NGS still showed a high sensitivity (81%) compared with the low sensitivity (14%) with direct sequencing.[36] Moreover, NGS showed actionable results in nearly one half of cases with no appreciable cellularity. Their findings suggested that NGS is both sensitive and specific for mutational analysis of EGFR in serous effusion cytology material and might have an advantage compared with other sequencing methods for cases in which the cellularity is low.[36]

Another gene of great importance in the management of lung cancer is ALK. It is located on the short arm of chromosome 2 and encodes a receptor tyrosine kinase belonging to the insulin receptor superfamily. It has an extracellular domain, which acts as a receptor, and an intracellular tyrosine kinase. When activated, this kinase will engage the JAK (janus kinase), PI3K (phosphoinositide 3-kinase)-AKT (protein kinase B), mTOR (mammalian target of rapamycin), sonic hedgehog, JUNB, CRKL-C3G, and MAPK (mitogen-activated protein kinase) pathways, resulting in increased survival and cellular growth. Its physiologic function is in fetal development, and it is not expressed by adult tissues, except in the brain. Several tumors will show activating mutations of the ALK or translocations that preserve the TK function.[37] Thus, ALK translocations will be present in 3% to 5% of NSCLC cases, and the guidelines have recommended that they be tested in all cases of advanced NSCLC.[25] Currently, 3 ALK inhibitors have been approved by the FDA, with more in clinical trials. These drugs also have an effect on ROS1 and MET. Crizotinib was the first ALK inhibitor to enter clinical practice. In patients with ALK-positive, treatment-naive disease, crizotinib has shown marked advantages compared with chemotherapy in the overall response rate and progression-free survival but not in overall survival. As it became the standard of care, the emergence of resistance was soon documented. Thus, new, more powerful inhibitors were developed, such as the so-called second-generation ALK inhibitors ceritinib and alectinib. They have shown activity against crizotinib-resistant lung cancer and remarkable activity against central nervous system metastasis. Alectinib has significantly fewer side effects compared with crizotinib. Several other ALK inhibitors are in phase I and II clinical trials.[38]

In tissue biopsy specimens, testing for ALK rearrangements using fluorescence in situ hybridization (FISH) or IHC has been recommended.[14] The use of these and other techniques has been thoroughly described for serous effusion cytology. Two studies reported on the successful detection of ALK translocations using FISH only.[39,40] Three others, 2 by the same investigators, compared FISH and IHC in serous effusion cell blocks.[41-43] They did not use matched tissue controls but found good concordance between the 2 techniques. In 2 of these studies, however, the incidence of ALK rearrangements was low compared with that reported in previous studies.[41-43] Three studies reported that the use of reverse transcription (RT)-PCR was possible for various types of specimens, including effusions.[44-46] One of them did not compare with other methods and the other 2 had used FISH or IHC as the reference standard. They concluded that concordance is high and that RT-PCR can be used for the detection of ALK fusions in specimens without adequate cellularity for FISH or IHC.[44-46]

In addition to EGFR, ALK, ROS1, and BRAF, the list of actionable targets in the lung has been expanding. For the purposes of prognostic stratification, investigational therapy, and enrollment in clinical trials, searches can be performed for other genomic alterations, including in RET, NRG1, MET, HER2, NF1, PIK3CA, FGFR2, NTRK1/2/3, and MEK1 genes.[25,47,48]

Using RT-PCR and pyrosequencing in a series of 102 patients, specimens were analyzed 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.[47] The results were matched with those from tissue biopsy specimens, with a global concordance of 88%.[47] One study reported on the successful detection of RET rearrangements using RT-PCR in effusion specimens of lung carcinomas in which RET status was known.[49]

Three studies considered the use of NGS on serous effusion specimens in the context of lung cancer for the purposes of multiplex analysis. One of them used an NGS assay for EGFR, KRAS, PIK3CA, BRAF, MET, HER2, ALK, ROS1, and RET.[50] It complemented these results with those from the amplification−refractory mutation system PCR for EGFR, ALK, and KRAS. Molecular tests were performed on cell block material, and tissue matched controls were used. The series was small, including only 30 patients.[50] They found comparable results between methods and between cell block material and tissue biopsy specimens, highlighting the advantages of NGS in the assessment of many different genes

using a limited amount of DNA.[50] Another study of 32 malignant effusion cell block samples performed DNA-based sequencing using NGS for KRAS, EGFR, BRAF, ROS1, ALK, RET, MET, ERBB2, PIK3CA, NRAS, AKT1, and MAP2K1 and other genes frequently mutated in lung cancer, such as TP53, STK11, KEAP1, CDKN2A, and RB1. They reported that the results between the cell blocks and tissue biopsy specimens were largely concordant and explained the few discrepancies with the discovery of resistance mutations.[51] One ALK case was missed by NGS on the pleural effusion owing to the presence of a noncanonical breakpoint fusion, highlighting the limitations of DNA-only sequencing.[51] One study considered cfDNA in the supernatant of pleural effusions, applying an NGS panel of 180 genes to 23 paired plasma and pleural cfDNA samples of patients with advanced lung cancer.[52] They found actionable mutations in 87% of effusion samples compared with 48% in plasma, showing a clear advantage for effusion samples compared with serum. Tissue controls were not used.[52]

A drug targeting NTRK1/2/3 gene fusions (larotrectinib) was approved by the FDA in 2018 for adult and pediatric patients with advanced solid tumors, including lung adenocarcinoma. Although specific studies for lung adenocarcinomas are lacking, the reported data showed that these fusions can effectively be searched using NGS in several sample types, including serous effusions.[48,53]

HER-2 and BRCA1/2 testing

HER2 is a gene located on the long arm of chromosome 17. It is a part of the EGFR/HER/ERBB family.[54] HER2 participates in intercellular signaling through signal transduction, similar to EGFR. Unlike EGFR, however, HER2 lacks a ligand. It is activated through dimerization with another member of its family. When it is amplified in breast cancer, spontaneous dimerization occurs, leading to constitutive activation and decoupling of cell proliferation and survival from an external stimulus.[55] This explains why breast cancers with HER2 amplification are more aggressive than their HER2-negative counterparts and why HER2 makes for an efficacious therapeutic target.[56] Because the first monoclonal antibody developed specifically against HER2, trastuzumab, was approved for clinical use in 1998, our knowledge and targeting of this molecule have progressed significantly.[57]

Five different drugs targeting HER2 are now available: 2 monoclonal antibodies (trastuzumab and pertuzumab) and 3 tyrosine kinase inhibitors (lapatinib, afatinib, and neratinib). They have been used to treat, not only the 20% to 25% of breast carcinoma cases that show positivity for HER2, but also gastroesophageal and colorectal cancer cases that overexpress HER2.[58-60]

HER2 testing has been standardized by the College of American Pathologists guidelines for tissue biopsies and surgical specimens. IHC can be used in a semiquantitative fashion, with in situ hybridization used to confirm equivocal results.[61] Breast, gastroesophageal, and colorectal cancers will frequently metastasize to serous cavities.[4] In the case of breast cancer, tissue biopsy specimens will usually be easy to obtain. However, in these tumors, HER2 status can sometimes be different between the primary and metastatic tumors, with prognostic significance.[61,62] In gastroesophageal and colorectal cancers, a serous effusion might be the first manifestation of disease and provide better material for molecular testing compared with endoscopic biopsy specimens.[63,64]

Four different studies have shown excellent concordance in the use of FISH for the determination of HER2 status between pleural effusion cytology in breast cancer, using both smears and cell blocks, and concomitant biopsy specimens of tissue metastasis.[65-68] Two of these also evaluated the use of IHC for determining HER2 status, concluding that this technique was also comparable between sample types.[65,66] One single, small series suggested this is also true for gastric adenocarcinoma.[69] Others have argued, however, that experience with HER2 IHC in effusions is fundamental, because the interpretation can be difficult owing to confusion between malignant and reactive mesothelial and inflammatory cells in cases with a markedly discohesive pattern without frank pleomorphism.[70] However, no studies have reported specifically on colorectal cancer, although the results should overlap with those found for other cancer types.

BRCA1 and BRCA2 are tumor suppressor genes located in the long arm of chromosomes 17 and 13, respectively. Their function is in the repair of chromosomal damage, in particular, of double strand breaks through homology-directed repair. Germline-inactivating mutations of these genes will lead to an increased risk of several cancers, most frequently, breast and ovarian cancer.[71] These mutations can also occur in the sporadic setting and have therapeutic implications in the context of both ovarian and breast carcinoma.[72] BRCA-mutated ovarian cancer and BRCA-mutated HER2-negative metastatic breast cancer can be targeted by poly-(ADP-ribose) polymerase (PARP) inhibitors. PARP is a nuclear protein involved in the recruitment of DNA repair proteins to the site of DNA breaks and its inhibition in BRCA-mutated neoplasms appears to lead to increased cell death through several complex mechanisms. As of 2019, 4 PARP inhibitors had been approved for use in monotherapy by the FDA: olaparib, rucaparib, niraparib, and talazoparib.[73] Because BRCA mutations can occur in both hereditary and sporadic settings, they should be tested for in tumor tissue samples. Traditionally, Sanger sequencing has been used; however, NGS has been shown to be equally effective and has been increasing in popularity.[74] Both breast carcinoma and high-grade serous carcinoma of the ovary will frequently metastasize to serous cavities. Data are lacking regarding BRCA testing for breast carcinoma using material obtained from serous effusions. However, a recent study has reported, in the context of high-grade serous carcinoma of the ovary, that 100% concordance seems to be present between cytologic and histologic samples, highlighting the viability of effusion cytology for genetic testing in this context.[75]

We would like to highlight the importance of the pathologist in guiding the process of molecular diagnostics and theranostics using serous effusions. At present, these tests require minimum cellularity. Furthermore, the use of these tests only makes sense in the context of a specific morphologic diagnosis. They cannot substitute for, but can only enhance the morphologic diagnosis. Given that new therapeutic targets are being discovered daily, the many drugs now in clinical trials and the minimally invasive process of obtaining serous effusion samples, we expect this area of research to be quite active in the near future.

Malignant mesothelioma: a quest for diagnosis on effusion cytology

The diagnosis of malignant mesothelioma using effusion cytology specimens has been controversial, and many pathologists have been trained never to make this diagnosis. A recent survey of 55 laboratories revealed that approximately two thirds will provide a definitive diagnosis of mesothelioma from an effusion specimen; however, one third will never do so.[76] An unequivocal diagnosis of mesothelioma is both feasible and accurate and can be helped by the use of a number of new tests, as described in the subsequent sections.

Clinical features

Clinical and, in particular, radiologic, information will often be ignored by pathologists but can be extremely helpful in this setting. Nodular pleural thickening found on computed tomography studies will usually be a sign of malignancy, although not specific to mesothelioma. Thus, the finding of small nodules on peritoneal surfaces is similarly typical of malignancy (Fig. 1A). Thickening of the mediastinal pleura will almost always indicate malignancy, as will omental caking. Smooth lateral pleural thickening can indicate malignancy but is not specific, and the same is true for omental or mesenteric stranding. Bilateral effusions are strong indication against a diagnosis of mesothelioma, but if malignant, favor metastatic disease.

Morphologic features of malignant mesothelioma in effusion specimens

As a rule, sarcomatous mesothelioma will not shed into effusions; thus, the cytologic diagnosis of mesothelioma will almost always pertain to epithelial tumors. The agreement in the reported data on the cytologic/morphologic features of mesotheliomas in effusion specimens has not been great. However, the 2 features that have been generally accepted are a highly cellular specimen and/or innumerable balls or papillary groups of atypical mesothelial cells, with individual balls/papillary groups containing upward of 10, 20, or 50 cells, depending on the investigator. Although numerous microscopic patterns of mesothelioma have been shown in tissue sections, these have not translated into the findings from effusion specimens in any simple way. However, the cells of mesotheliomas tend to be much more monotonous than those of carcinomas, and mesotheliomas will often be deceptively bland appearing.

Separation of mesothelioma from metastatic carcinoma

Historically, the determination that atypical cells were mesothelial instead of metastatic carcinoma using liquid-based preparations and Papanicolaou stains has been an impediment to the diagnosis of mesothelioma. The traditional approach has been to determine whether the malignant cells are similar to benign mesothelial cells or form a separate population. This distinction can be difficult and often inaccurate using Papanicolaou stains. However, more importantly, the method has become outmoded, because (provided one has a cell block), the determination that cells are mesothelial is a straightforward application of IHC. The use of Papanicolaou staining should be reserved for deciding whether the cells in question are malignant, which can also, sometimes, be easier using cell blocks (Fig. 1B, C).

A useful starting point for IHC is to test for 2 mesothelial markers and 2 carcinoma markers. Unless reason exists to believe that a given specimen has been derived from a carcinoma of a known primary site, a broad-spectrum carcinoma marker should be one of the stains used. This will be true even for cases that appear to mark as mesothelial. Claudin-4 is probably the best broad-spectrum marker for this setting because it will not cross react with mesothelial cells (Fig. 1D).[77] MOC-31 can cross react with mesothelial cells; this phenomenon appears to be, at least in part, laboratory dependent. BerEP4 has known cross reactivity with mesothelial cells and should be avoided when the issue is determining mesothelioma versus metastatic carcinoma. CEA (carcinoembryonic antigen), CD15, and B72.3 have low sensitivity and a degree of cross reactivity with mesothelial cells and have been outmoded as broad-spectrum carcinoma markers.

Calretinin, cytokeratin 5/6, WT-1, D2-40, and mesothelin are good choices as mesothelial markers (Fig. 1E-G); which marker to pick depends on the likely differential diagnosis and the origin of the sample. WT-1, for example, is not a good choice in the peritoneal cavity in women, because a high proportion of gynecologic malignancies will stain with WT-1. Virtually all epithelial mesothelioma cases will stain with calretinin, and caution should be exercised before determining a specimen is positive for mesothelioma if calretinin staining is negative. However, none of these markers will be 100% specific for mesothelioma, and most will stain with some forms of carcinoma. Hence, the recommendation has been to use $\geq$2 mesothelial markers and a broad-spectrum carcinoma marker.

The method of fixation of the specimen will greatly affect the immunoreactivity. Alcohol-based fixatives such as CytoLyt will leach many antigens in a generally time dependent, but often, unpredictable fashion. Specimens fixed only in formalin will provide much more reliable IHC. If the cell blocks of alcohol-fixed material are used, separate validation of the IHC procedures is required.

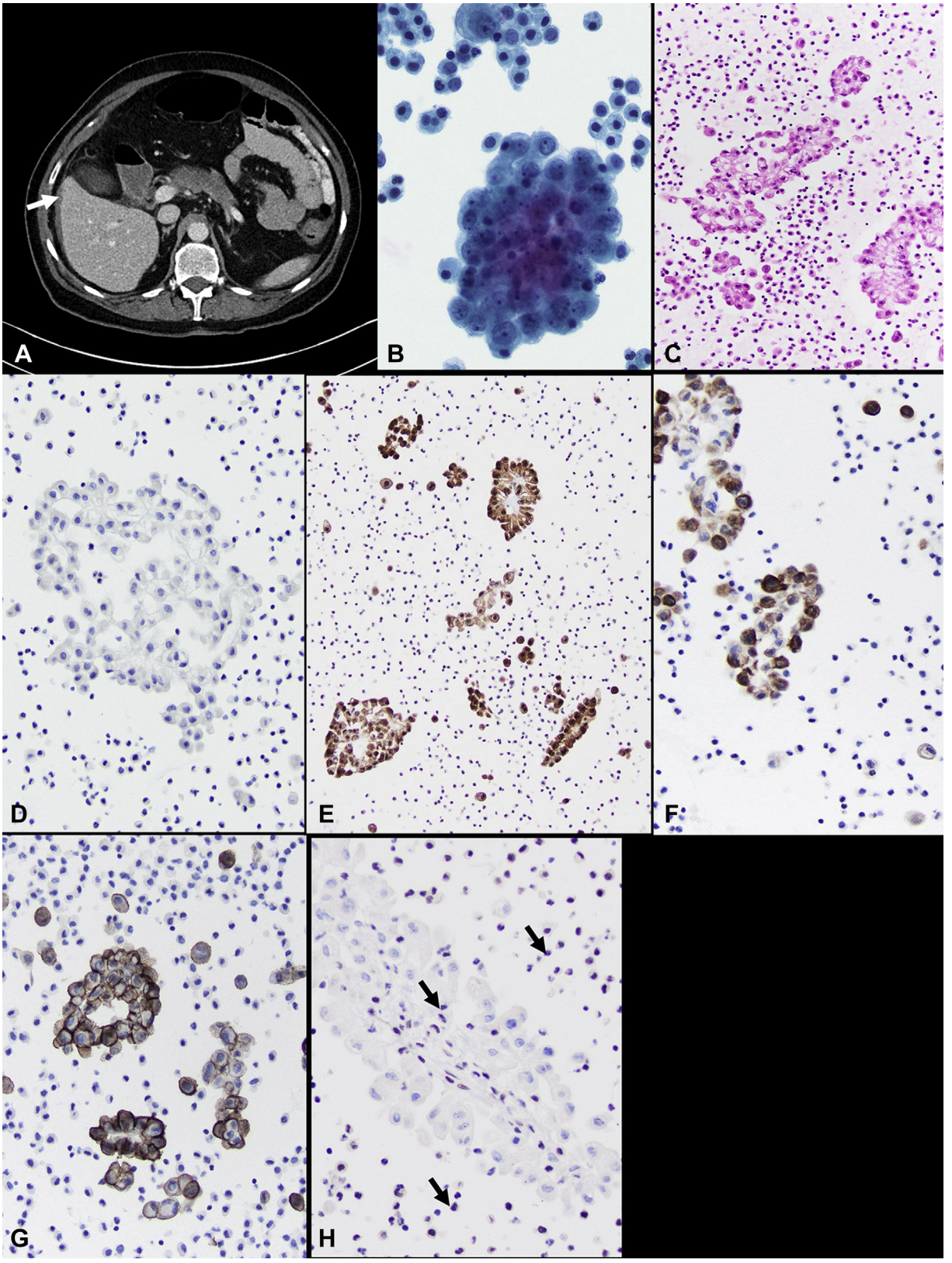

Figure 1 Peritoneal malignant mesothelioma diagnosed from an effusion specimen. A woman in her 50s had presented with abdominal swelling. (A), Computed tomography scan showing ascites and subtle nodularity along the liver (arrow) suggestive of tumor. (B), Papanicolaou stain of the effusion specimen showing balls of malignant cells. (C), In the cell block, numerous papillary groups can be seen. (D), Staining for the broad-spectrum carcinoma marker, claudin-4, is negative. Stains for calretinin (E), CK5/6 (F), and D2-40 (G) were positive, confirming these were mesothelial cells. (H) Stain for BAP1 showing nuclear loss, an indicator of malignancy. Arrows point to positive inflammatory cells, which served as an internal control.

Separation of benign from malignant mesothelial proliferation

The separation of benign and malignant mesothelial proliferations can frequently be difficult, not only for cytology specimens but also for tissue biopsy specimens. In effusions, the same basic morphology that defines a mesothelioma (ie, high cellularity and/or innumerable balls/papillary clusters of atypical mesothelial cells) can also serve to separate mesothelioma from benign reactions. However, some cases of mesothelioma will not demonstrate these features, and, in addition, reactive mesothelial cells can be quite atypical.

A variety of adjunctive techniques have been proposed for aiding in this separation. IHC stains such as desmin, EMA, p53, IMP-3, and Glut-1 can work statistically when reviewing large numbers of cases. However, too many cases will stain "incorrectly" to consider these stains reliable for individual cases. In contrast, 3 new markers based on molecular abnormalities found in mesotheliomas are available, and these appear to be reliable for differentiating mesotheliomas from reactive processes.[78-80] *BAP1, MTAP* (methylthioadenosine phosphorylase), and *CDKN2A* (often referred to as "p16") are all believed to be tumor suppressor genes. In malignant mesotheliomas, the feature to look for is the loss of their expression. Benign tissues will show nuclear BAP1 by IHC; cytoplasmic and, sometimes, nuclear MTAP by IHC; and 2 signals/nucleus for *CDKN2A* by FISH (CDKN2A IHC will not provide the same results as FISH and should not be used in this setting).

The loss of nuclear BAP1 staining should always be considered indicative of malignancy (Fig. 1H).[81] For MTAP, the loss of cytoplasmic staining is an indicator of malignancy.[80] For both markers, a positive internal control of inflammatory cells should always be included (Fig. 1H). In the absence of such a control, the stains will not always be interpretable. For *CDKN2A* FISH, the reported standards have used tissue sections with homozygous loss. However, because tissue sections will be much thinner than nuclei, some proportion of normal cells will always appear to have lost 1 or both *CDKN2A* copies. To allow for this truncation artifact, it will necessary to run a series of cases to determine the "background" range of truncation loss and to count 50 to 100 cells for a given test case.[78,80] CKDN2A FISH can also be used on smears and cytocentrifuge preparations and will be easier to interpret because no truncation effect will be present.

The limitation for all 3 of these tests has been their sensitivity, which, for epithelial mesotheliomas in the pleural cavity, has been ~60% for each marker. Increased sensitivity can be achieved by running 2 tests,[80,82] with a resulting sensitivity in the range of 80% to 90%. Also, because good correlation has been found for MTAP loss using IHC and *CDKN2A* loss using FISH,[79,80] performing both BAP1 and MTAP IHC will probably be the most efficient approach. In addition, *CDKN2A* loss will be much less frequent in peritoneal mesothelioma, which is probably also true for MTAP loss, because these genes will be commonly codeleted.

Conclusions

The diagnosis of malignant mesothelioma in effusion specimens is accurate and reliable when the morphologic findings are typical. In equivocal cases, the separation of mesothelioma from reactive mesothelial proliferations can be aided by testing for 3 new markers: BAP1 and MTAP using IHC and *CDKN2A* using FISH.

Funding sources

No specific funding was disclosed.

Conflict of interest disclosures

The authors made no disclosures.

References

1. Krausz T, McGregor SM. The mesothelium. In: Marchevsky AM, Husain AN, Galateau-Sallé F, eds. *Practical Pathology of Serous Membranes*. Cambridge, UK: Cambridge University Press; 2019:1–9.
2. Thomsen TW, DeLaPena J, Setnik GS. Videos in clinical medicine: thoracentesis. *N Engl J Med*. 2006;355:e16.
3. Mazurek JM, Syamlal G, Wood JM, Hendricks SA, Weston A. Malignant mesothelioma mortality—United States, 1999-2015. *MMWR Morb Mortal Wkly Rep*. 2017;66:214–218.
4. Cibas ES, Ducatman BS. *Cytology: Diagnostic Principles and Clinical Correlates*. New York: Elsevier Health Sciences Division; 2014.
5. Nance KV, Shermer RW, Askin FB. Diagnostic efficacy of pleural biopsy as compared with that of pleural fluid examination. *Mod Pathol*. 1991;4:320–324.
6. Wolfe KS, Kress JP. Risk of procedural hemorrhage. *Chest*. 2016;150:237–246.
7. Corcoran JP, Psallidas I, Wrightson JM, Hallifax RJ, Rahman NM. Pleural procedural complications: prevention and management. *J Thorac Dis*. 2015;7:1058–1067.
8. Altman E, Spiridonov L, Vadim S, Alejandro L, Alexei G, Hector CI. Efficacy of cytology, cell blocks and thoracoscopic pleural biopsy in malignant pleural effusion diagnosis. *Eur Respir J*. 2013;42:P3073.
9. Husain H, Nykin D, Bui N, et al. Cell-free DNA from ascites and pleural effusions: molecular insights into genomic aberrations and disease biology. *Mol Cancer Ther*. 2017;16:948–955.
10. Roh MH. The utilization of cytologic and small biopsy samples for ancillary molecular testing [e-pub ahead of print]. *Mod Pathol*; 2019. https://doi.org/10.1038/s41379-018-0138-z. [Epub ahead of print].
11. Alì G, Bruno R, Fontanini G. The pathological and molecular diagnosis of malignant pleural mesothelioma: a literature review. *J Thorac Dis*. 2018;10:S276–S284.
12. Kinoshita Y, Hida T, Hamasaki M, et al. A combination of MTAP and BAP1 immunohistochemistry in pleural effusion cytology for the diagnosis of mesothelioma. *Cancer Cytopathol*. 2018;126:54–63.
13. Schmitt FC. *Molecular Applications in Cytology*. New York: Springer International Publishing; 2018.
14. Lindeman NI, Cagle PT, Aisner DL, et al. Updated molecular testing guideline for the selection of lung cancer patients for treatment with targeted tyrosine kinase inhibitors: guideline from the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology. *Arch Pathol Lab Med*. 2018;142:321–346.
15. European Molecular Biology Laboratory–Bioinformatics Institute. EGFR (human gene). Ensembl. Available at: https://www.ensembl.

org/Homo_sapiens/Gene/Summary?g=ENSG00000146648;r=7:55019017-55211628;redirect=no. Accessed August 30, 2019.
16. Wieduwilt MJ, Moasser MM. The epidermal growth factor receptor family: biology driving targeted therapeutics. *Cell Mol Life Sci*. 2008;65:1566–1584.
17. Singh M, Jadhav HR. Targeting non-small cell lung cancer with small-molecule EGFR tyrosine kinase inhibitors. *Drug Discov Today*. 2018;23:745–753.
18. Hanna N, Johnson D, Temin S, et al. Systemic therapy for stage IV non-small-cell lung cancer: American Society of Clinical Oncology clinical practice guideline update. *J Clin Oncol*. 2017;35:3484–3515.
19. Sheikine Y, Rangachari D, McDonald DC, et al. EGFR testing in advanced non-small-cell lung cancer, a mini-review. *Clin Lung Cancer*. 2016;17:483–492.
20. Joshi A, Zanwar S, Noronha V, et al. EGFR mutation in squamous cell carcinoma of the lung: does it carry the same connotation as in adenocarcinomas? *Onco Targets Ther*. 2017;10:1859–1863.
21. Ellison G, Zhu G, Moulis A, Dearden S, Speake G, McCormack R. EGFR mutation testing in lung cancer: a review of available methods and their use for analysis of tumour tissue and cytology samples. *J Clin Pathol*. 2013;66:79–89.
22. Wetterstrand KA. DNA sequencing costs: data from the National Hormone Genome Research Institute Genome Sequencing Program (GSP). Available at: https://www.genome.gov/about-genomics/fact-sheets/DNA-Sequencing-Costs-Data. Accessed August 30, 2019.
23. McCombie WR, McPherson JD, Mardis ER. Next-generation sequencing technologies [e-pub ahead of print]. *Cold Spring Harbor Perspect Med*; 2018. https://doi.org/10.1101/cshperspect.a025783. [Epub ahead of print].
24. Schwarze K, Buchanan J, Taylor JC, Wordsworth S. Are whole-exome and whole-genome sequencing approaches cost-effective? A systematic review of the literature. *Genet Med*. 2018;20:1122–1130.
25. Kalemkerian GP, Narula N, Kennedy EB, et al. Molecular testing guideline for the selection of patients with lung cancer for treatment with targeted tyrosine kinase inhibitors: American Society of Clinical Oncology endorsement of the College of American Pathologists/International Association for the Study of Lung Cancer/Association for Molecular Pathology clinical practice guideline update. *J Clin Oncol*. 2018;36:911–919.
26. Guan Y, Wang ZJ, Wang LQ, Hua DF, Liu J. Comparison of EGFR mutation rates in lung adenocarcinoma tissue and pleural effusion samples. *Genet Mol Res*. 2016;15. https://doi.org/10.4238/gmr.15027001.
27. Sun PL, Jin Y, Kim H, Lee CT, Jheon S, Chung JH. High concordance of EGFR mutation status between histologic and corresponding cytologic specimens of lung adenocarcinomas. *Cancer Cytopathol*. 2013;121:311–319.
28. Yang J, Lee O-J, Son S-M, et al. EGFR mutation status in lung adenocarcinoma-associated malignant pleural effusion and efficacy of EGFR tyrosine kinase inhibitors. *Cancer Res Treat*. 2018;50:908–916.
29. Yeo CD, Kim JW, Kim KH, et al. Detection and comparison of EGFR mutations in matched tumor tissues, cell blocks, pleural effusions, and sera from patients with NSCLC with malignant pleural effusion, by PNA clamping and direct sequencing. *Lung Cancer*. 2013;81:207–212.
30. Lin J, Gu Y, Du R, Deng M, Lu Y, Ding Y. Detection of EGFR mutation in supernatant, cell pellets of pleural effusion and tumor tissues from non-small cell lung cancer patients by high resolution melting analysis and sequencing. *Int J Clin Exp Pathol*. 2014;7:8813–8822.
31. Lee JS, Hur JY, Kim IA, et al. Liquid biopsy using the supernatant of a pleural effusion for EGFR genotyping in pulmonary adenocarcinoma patients: a comparison between cell-free DNA and extracellular vesicle-derived DNA. *BMC Cancer*. 2018;18:1236.
32. Wang W, Tang Y, Li J, Zhang Y, Zou Y, Su X. Identification of EGFR mutations in cytological specimens of non-small cell lung carcinoma from a single institute. *Int J Clin Exp Pathol*. 2018;11:929–935.
33. Goto K, Satouchi M, Ishii G, et al. An evaluation study of EGFR mutation tests utilized for non-small-cell lung cancer in the diagnostic setting. *Ann Oncol*. 2012;23:2914–2919.
34. Rossi E, Bizzarro T, Martini M, et al. The role of liquid based cytology and ancillary techniques in the peritoneal washing analysis: our institutional experience. *PLoS One*. 2017;12:e0168625.
35. Rossi ED, Bizzarro T, Schmitt F, Longatto-Filho A. The role of liquid-based cytology and ancillary techniques in pleural and pericardiac effusions: an institutional experience. *Cancer Cytopathol*. 2015;123:258–266.
36. Buttitta F, Felicioni L, Del Grammastro M, et al. Effective assessment of EGFR mutation status in bronchoalveolar lavage and pleural fluids by next-generation sequencing. *Clin Cancer Res*. 2013;19:691–698.
37. Zhao Z, Verma V, Zhang M. Anaplastic lymphoma kinase: role in cancer and therapy perspective. *Cancer Biol Ther*. 2015;16:1691–1701.
38. Rothenstein JM, Chooback N. ALK inhibitors, resistance development, clinical trials. *Curr Oncol*. 2018;25:S59–S67.
39. Carter J, Miller JA, Feller-Kopman D, Ettinger D, Sidransky D, Maleki Z. Molecular profiling of malignant pleural effusion in metastatic non–small-cell lung carcinoma: the effect of preanalytical factors. *Ann Am Thorac Soc*. 2017;14:1169–1176.
40. Cai G, Wong R, Chhieng D, et al. Identification of EGFR mutation, KRAS mutation, and ALK gene rearrangement in cytological specimens of primary and metastatic lung adenocarcinoma. *Cancer Cytopathol*. 2013;121:500–507.
41. Wang W, Tang Y, Li J, Jiang L, Jiang Y, Su X. Detection of ALK rearrangements in malignant pleural effusion cell blocks from patients with advanced non-small cell lung cancer: a comparison of Ventana immunohistochemistry and fluorescence in situ hybridization. *Cancer Cytopathol*. 2015;123:117–122.
42. Wang W-Y, Liang D-N, Yao W-Q, et al. Immunohistochemical screening and fluorescence in situ hybridization confirmation of ALK translocation in lung adenocarcinoma and its clinicopathological significance: a single-center large-scale investigation of Chinese patients. *Hum Pathol*. 2014;45:1414–1422.
43. Liu L, Zhan P, Zhou X, et al. Detection of EML4-ALK in lung adenocarcinoma using pleural effusion with FISH, IHC, and RT-PCR methods. *PLoS One*. 2015;10:e0117032.
44. Soda M, Isobe K, Inoue A, et al. A prospective PCR-based screening for the EML4-ALK oncogene in non–small cell lung cancer. *Clin Cancer Res*. 2012;18:5682.
45. Wu S-G, Kuo Y-W, Chang Y-L, et al. EML4-ALK translocation predicts better outcome in lung adenocarcinoma patients with wild-type EGFR. *J Thorac Oncol*. 2012;7:98–104.
46. Chen YL, Lee CT, Lu CC, et al. Epidermal growth factor receptor mutation and anaplastic lymphoma kinase gene fusion: detection in malignant pleural effusion by RNA or PNA analysis. *PLoS One*. 2016;11:e0158125.
47. Akamatsu H, Koh Y, Kenmotsu H, et al. Multiplexed molecular profiling of lung cancer using pleural effusion. *J Thorac Oncol*. 2014;9:1048–1052.
48. Roy-Chowdhuri S, Pisapia P, Salto-Tellez M, et al. Invited review—next-generation sequencing: a modern tool in cytopathology. *Virchows Arch*. 2019;475:3–11.
49. Tsai T-H, Wu S-G, Hsieh M-S, Yu C-J, Yang JC-H, Shih J-Y. Clinical and prognostic implications of RET rearrangements in metastatic lung adenocarcinoma patients with malignant pleural effusion. *Lung Cancer*. 2015;88:208–214.
50. Liu L, Shao D, Deng Q, et al. Next generation sequencing-based molecular profiling of lung adenocarcinoma using pleural effusion specimens. *J Thorac Dis*. 2018;10:2631–2637.
51. Yang SR, Lin CY, Stehr H, et al. Comprehensive genomic profiling of malignant effusions in patients with metastatic lung adenocarcinoma. *J Mol Diagn*. 2018;20:184–194.
52. Zhou Z, Chen R, Chu Y, et al. Lung cancer genomic alterations in cell free DNA in pleural effusion compared to plasma. *J Clin Oncol*. 2017;35:e23206.

53. Huang FW, Feng FY. A tumor-agnostic NTRK (TRK) inhibitor. *Cell*. 2019;177:8.
54. European Molecular Biology Laboratory—Bioinformatics Institute. ERBB2 (human gene). Ensembl. Available at: https://www.ensembl.org/Homo_sapiens/Gene/Summary?g=ENSG00000141736;r=17:39687914-39730426. Accessed August 30, 2019.
55. Ruiz-Saenz A, Moasser MM. Targeting HER2 by combination therapies. *J Clin Oncol*. 2018;36:808—811.
56. Ross JS, Fletcher JA, Linette GP, et al. The Her-2/neu gene and protein in breast cancer 2003: biomarker and target of therapy. *Oncologist*. 2003;8:307—325.
57. Lakhtakia R, Burney I. A brief history of breast cancer: part III—tumour biology lays the foundation for medical oncology. *Sultan Qaboos Univ Med J*. 2015;15:e34—e38.
58. Meisel JL, Venur VA, Gnant M, Carey L. Evolution of targeted therapy in breast cancer: where precision medicine began. *Am Soc Clin Oncol Educ Book*. 2018;38:78—86.
59. Lordick F, Janjigian YY. Clinical impact of tumour biology in the management of gastroesophageal cancer. *Nat Rev Clin Oncol*. 2016;13:348—360.
60. Graham DM, Coyle VM, Kennedy RD, Wilson RH. Molecular subtypes and personalized therapy in metastatic colorectal cancer. *Curr Colorectal Cancer Rep*. 2016;12:141—150.
61. Wolff AC, Hammond MEH, Allison KH, et al. Human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline focused update. *Arch Pathol Lab Med*. 2018;142:1364—1382.
62. Yeung C, Hilton J, Clemons M, et al. Estrogen, progesterone, and HER2/neu receptor discordance between primary and metastatic breast tumours—a review. *Cancer Metastasis Rev*. 2016;35:427—437.
63. Maeda H, Kobayashi M, Sakamoto J. Evaluation and treatment of malignant ascites secondary to gastric cancer. *World J Gastroenterol*. 2015;21:10936—10947.
64. Yuan Y, Ye J, Chen C, et al. Refractory left pleural effusion in an older patient: atypical presentation of colorectal carcinoma. *Oncol Lett*. 2015;9:1055—1058.
65. Shabaik A, Lin G, Peterson M, et al. Reliability of Her2/neu, estrogen receptor, and progesterone receptor testing by immunohistochemistry on cell block of FNA and serous effusions from patients with primary and metastatic breast carcinoma. *Diagn Cytopathol*. 2011;39:328—332.
66. Schluter B, Gerhards R, Strumberg D, Voigtmann R. Combined detection of Her2/neu gene amplification and protein overexpression in effusions from patients with breast and ovarian cancer. *J Cancer Res Clin Oncol*. 2010;136:1389—1400.
67. Ogawa K, Tominaga K, Oda M, et al. Discordant HER2 status between primary breast carcinoma and recurrent/metastatic tumors using fluorescence in situ hybridization on cytological samples. *Jpn J Clin Oncol*. 2012;43:55—62.
68. Nakayama Y, Nakagomi H, Omori M, et al. Benefits of using the cell block method to determine the discordance of the HR/HER2 expression in patients with metastatic breast cancer. *Breast Cancer*. 2016;23:633—639.
69. Bozzetti C, Negri FV, Lagrasta CA, et al. Comparison of HER2 status in primary and paired metastatic sites of gastric carcinoma. *Br J Cancer*. 2011;104:1372—1376.
70. Wong DD, de Boer WB, Platten MA, Jo VY, Cibas ES, Kumarasinghe MP. HER2 testing in malignant effusions of metastatic gastric carcinoma: is it feasible? *Diagn Cytopathol*. 2015;43:80—85.
71. Friedenson B. The BRCA1/2 pathway prevents hematologic cancers in addition to breast and ovarian cancers. *BMC Cancer*. 2007;7:152.
72. Neff RT, Senter L, Salani R. BRCA mutation in ovarian cancer: testing, implications and treatment considerations. *Ther Adv Med Oncol*. 2017;9:519—531.
73. Faraoni I, Graziani G. Role of BRCA mutations in cancer treatment with poly(ADP-ribose) polymerase (PARP) inhibitors. *Cancers (Basel)*. 2018;10:487.
74. Park HS, Park S-J, Kim JY, et al. Next-generation sequencing of BRCA1/2 in breast cancer patients: potential effects on clinical decision-making using rapid, high-accuracy genetic results. *Ann Surg Treat Res*. 2017;92:331—339.
75. Gornjec A, Novakovic S, Stegel V, et al. Cytology material is equivalent to tumor tissue in determining mutations of BRCA 1/2 genes in patients with tubo-ovarian high grade serous carcinoma. *BMC Cancer*. 2019;19:296.
76. Paintal A, Raparia K, Zakowski MF, Nayar R. The diagnosis of malignant mesothelioma in effusion cytology: a reappraisal and results of a multi-institution survey. *Cancer Cytopathol*. 2013;121:703—707.
77. Ordóñez NG. Broad-spectrum immunohistochemical epithelial markers: a review. *Hum Pathol*. 2013;44:1195—1215.
78. Churg A, Sheffield BS, Galateau-Salle F. New markers for separating benign from malignant mesothelial proliferations: are we there yet? *Arch Pathol Lab Med*. 2016;140:318—321.
79. Hida T, Hamasaki M, Matsumoto S, et al. Immunohistochemical detection of MTAP and BAP1 protein loss for mesothelioma diagnosis: comparison with 9p21 FISH and BAP1 immunohistochemistry. *Lung Cancer*. 2017;104:98—105.
80. Berg KB, Dacic S, Miller C, Cheung S, Churg A. Utility of methylthioadenosine phosphorylase compared with BAP1 immunohistochemistry, and CDKN2A and NF2 fluorescence in situ hybridization in separating reactive mesothelial proliferations from epithelioid malignant mesotheliomas. *Arch Pathol Lab Med*. 2018;142:1549—1553.
81. Wang LM, Shi ZW, Wang JL, et al. Diagnostic accuracy of BRCA1-associated protein 1 in malignant mesothelioma: a meta-analysis. *Oncotarget*. 2017;8:68863—68872.
82. Hwang HC, Sheffield BS, Rodriguez S, et al. Utility of BAP1 immunohistochemistry and p16 (CDKN2A) FISH in the diagnosis of malignant mesothelioma in effusion cytology specimens. *Am J Surg Pathol*. 2016;40:120—126.