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Biomarkers of malignancy in pleural effusions

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ABSTRACT

The measurement of biomarkers in pleural fluid plays a crucial role in the diagnosis of malignant pleural effusion. Four types of biomarkers are discussed in this review: (1) soluble protein biomarkers, such as combined carcinoembryonic antigen and carbohydrate antigen 15-3, which can provide valuable insights into the malignant nature of an effusion, particularly those with false-negative cytology; (2) immunocytochemical biomarkers, which help establish the mesothelial, epithelial, or other cell origin of a malignant effusion, as well as the primary tumor invading the pleura (e.g., epithelial cell adhesion molecule and claudin-4 in carcinomas, or loss of BRCA1-associated protein 1 and methylthioadenosine phosphorylase in mesotheliomas); (3) flow cytometry biomarkers, which identify lymphoproliferative as well as epithelial neoplasms; and (4) molecular biomarkers (e.g., oncogenic or driver mutations), which phenotypically characterize a tumor and predict its response to target therapy or immunotherapy.

Keywords: Cancer biomarkers. Lung cancer. Malignant pleural effusion. Pleural fluid.

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INTRODUCTION

Pleural effusions can result from more than 60 different conditions although four etiologies account for nearly 80% of all cases seen in clinical practice: heart failure, malignancy, pneumonia, and tuberculosis[1]. Biomarkers, which are molecules derived from biological processes, can be used to identify the underlying cause of pleural effusion. They are measured in pleural fluid and have been incorporated as an essential tool for the rapid, noninvasive assessment of this condition. The more difficult it is to diagnose a disease, the more sense it makes to use biomarkers. For example, by integrating biomarkers into diagnostic workflows, clinicians can improve the accuracy of malignant pleural effusion (MPE) identification. This review summarizes the main pleural fluid biomarkers of malignancy that are currently used in routine practice or have compelling evidence for future implementation.

BIOMARKERS IN MALIGNANT PLEURAL EFFUSIONS

MPE is the most common cause of pleural exudates. Three factors may hamper the diagnosis of MPE. First, the results of pleural fluid analysis may indicate a transudate based on Light's criteria[2], thereby redirecting the diagnostic algorithm. In a study of 1527 consecutive patients with MPE, of whom 62% had a definite diagnosis and 38% had a probable MPE (i.e., they were considered to have a false-negative pleural fluid cytological examination), 29 (1.9%) had fluids that met the transudative criteria[3]. Specifically, 20 (2.1%) definite MPEs and 9 (1.5%) probable MPEs were categorized as transudates. In most cases, there was a concomitant circumstance that could explain the presence of a malignant transudate, such as severe hypoalbuminemia or a nonexpandable lung. Second, and linked to this fact, pleural effusions secondary to dual or multiple etiologies are not infrequent. One study supported this, demonstrating that 30% of 126 patients with unilateral effusions had more than one underlying etiology[4]. In particular, of the 58 patients with a diagnosis of malignancy, 12 (21%) had multiple apparent causes contributing to the accumulation of pleural fluid. Heart failure was the most common secondary cause; thus, we may observe patients with exudative MPEs and elevated pleural fluid natriuretic peptide levels, indicating a partial contribution of heart failure to the development of pleural effusion. In brief, the establishment of a diagnosis does not exclude other causes. The third and most important factor hindering the identification of MPE is the moderate sensitivity of pleural fluid cytology. A meta-analysis of 36 studies, totaling 6,057 patients with MPE, revealed that the pooled diagnostic sensitivity of pleural fluid cytology was 58.2%[5]. However, this percentage differed significantly depending on the primary tumor type, with the highest sensitivity being observed in lung adenocarcinoma (83.6%) and the lowest in lung squamous cell lung cancer (24.2%) and mesothelioma (28.9%). Therefore, the main motivation for pursuing diagnostic biomarkers in pleural fluid is the limited sensitivity of the cytological analysis.

Accurate diagnosis of MPEs can be challenging, even when patients exhibit positive pleural fluid cytology, because cytomorphological findings may not be definitive for a particular tumor. Moreover, there is a current need for phenotype neoplasms to indicate targeted therapies or immunotherapy. Consequently, biomarkers used in MPEs can be classified into four distinct categories: (1) soluble biomarkers, composed of proteins that can be measured in pleural fluid supernatants, which are utilized to establish the malignant nature of the pleural effusion; (2) immunocytochemical biomarkers, which provide information about the primary tumor that invades the pleura; (3) flow cytometry markers, which are mainly used in the diagnosis and characterization of hematological cancers; and (4) molecular biomarkers, intended to detect genetic alterations with therapeutic implications.

SOLUBLE-PROTEIN BIOMARKERS

Many studies have investigated the diagnostic accuracy of conventional cancer biomarkers for labeling MPEs, including carcinoembryonic antigen (CEA), carbohydrate antigen 15-3 (CA15-3), carbohydrate antigen 125 (CA125), cytokeratin fragment antigen 21-1 (CYFRA 21-1), carbohydrate antigen 19-9 (CA 19-9), and neuron-specific enolase (NSE)[6]. Overall, the most accurate one is pleural fluid CEA, whereas the best tumor marker combination is CEA and CA 15-3 (a positive result indicated by the elevation of either marker)[7,8]. The sensitivity of these tumor markers is approximately 50% and the specificity is generally greater than 90% (Table 1)[9-14]. It should be noted that the evaluation of tumor markers for diagnostic purposes is often subject to shortcomings. First, the majority of studies establish threshold values with elevated specificity, but not absolute specificity. However, for the diagnostic usefulness of tumor markers, the selected cutoff points must be 100% specific (i.e., they cannot be surpassed by any benign effusion), even though this renders the tests less sensitive. Second, the composition of the benign comparator group influences the operating characteristics of tumor markers. For example, the inclusion of effusions easily recognized in clinical practice, such as empyemas or cardiac transudates, may lead to an overestimation of true test diagnostic accuracy. Finally, the main interest of pleural fluid tumor markers is to uncover the malignant nature of a pleural effusion in cases where a cytological examination has yielded a false-negative result. This is particularly relevant because for individuals with positive pleural fluid cytology, the diagnosis has already been confirmed, and thus, the clinical value of tumor markers is nil.

In a single-center series of 1,575 patients with nonpurulent pleural exudates, a clinical scenario in which malignancy should be ruled out, we tested the accuracy of electrochemiluminescence CEA and CA 15-3 levels in pleural fluid samples[15]. Based on threshold values with 100% specificity, it was found that 41, 40, and 60% of MPE cases exhibited pleural fluid levels of CEA > 45 ng/ml, CA 15-3 > 77 IU/l, or both, respectively. These percentages were 30, 19, and 41%, respectively, in patients with MPE demonstrated by pleural biopsy, whose pleural

Table 1. Meta-analyses of classical pleural fluid soluble biomarkers of malignancy

Biomarker	*N* (studies)	Sensitivity (%)	Specificity (%)	Positive LR	Negative LR	AUC
CEA[10]	36	55	96	14.4	0.47	0.81
CA 15-3[11]	21	58	91	8.93	0.46	0.84
CA 19-9[10]	8	38	98	18.8	0.64	0.91
CA 125[10]	10	57	93	7.99	0.46	0.79
CYFRA 21-1[10]	16	62	93	9.19	0.40	0.84
NSE[12]	14	53	85	3.54	0.56	0.78
Survivin[13]	10	86	92	8.76	0.16	0.94
CEA + CA 15-3[14]	4	64	98	28.1	0.37	0.88
CEA + CA 19-9[14]	4	58	98	32.3	0.43	0.96
CEA + CYFRA 21-1[14]	7	82	92	8.66	0.18	0.95
CA 15-3 + CYFRA 21-1[14]	4	88	94	8.83	0.06	0.98

AUC: area under the curve; CA: carbohydrate antigen; CEA: carcinoembryonic antigen; CYFRA: cytokeratin fragment antigen 21-1; LR: likelihood ratio; NSE: neuron-specific enolase.

fluid cytological examination produced false-negative results. Thus, more than one-third of cytology-negative MPE cases can be detected using at least one of these markers. Nevertheless, even with high pleural fluid concentrations of tumor markers, further confirmatory cytohistological diagnosis is mandatory.

Numerous additional cancer biomarkers have been explored in small patient cohorts, for which the available evidence is significantly less robust compared to the conventional biomarkers previously discussed. Examples provided in Table 2 include carbohydrate antigen 72-4 (CA 72-4)[16], human epididymis secretory protein 4 (HE4)[17], serum carbohydrate antigen 50 (CA50)[18], pro-gastrin-releasing peptide (proGRP)[19], calprotectin[20], and epithelial cell adhesion molecule (EpCAM)[21].

Biomarker concentrations in pleural fluid have also been linked to MPE prognosis. For example, in a study involving 224 patients with confirmed MPE, those with pleural fluid CA125 levels greater than 1,000 U/ml and CYFRA 21-1 levels greater than 100 ng/ml survived for 7 months less than those with lower levels[22], The prognostic value of survivin, a molecule that inhibits apoptosis, was examined in a cohort of 84 patients with MPE[23]. Patients with pleural fluid levels exceeding 30 pg/ml had a median survival of 4 months, whereas those below this threshold exhibited a median survival of 13 months. The poor prognosis of high pleural fluid survivin levels was subsequently validated through a meta-analysis of five studies involving 306 individuals, yielding a hazard ratio of 2.90[24].

Table 2. Other pleural fluid soluble biomarkers of malignancy

Biomarker	*N* (patients)	Sensitivity (%)	Specificity (%)	Positive LR	Negative LR	AUC	Comments
CA 72-4 > 8 µmol/l[16]	211	40	98	23.9	0.61	0.80	Values > 15 µmol/l (100% specificity) were observed in 17% of cytology-negative MPEs.
HE4 > 1,300 pmol/l[17]	210	47	91	ND	ND	0.75	In MPEs with negative cytology, the test had 27% sensitivity and 91% specificity.
CA50 > 15 IU/ml[18]	153	30	100	—	0.70	0.72	10% of cytology-negative MPEs exhibited high levels of CA50.
proGRP > 40 pg/ml (for SCLC)[19]	211 (6 SCLC)	100	59	2.61	0	0.90	proGRP had no diagnostic value for MPE in general, but was of diagnostic value for SCLC in particular.
Calprotectin < 6,233 ng/ml[20]	360	96	60	2.4	0.06	0.85	In patients with cytology-negative MPEs, the test had 97% sensitivity and 60% specificity.
EpCAM > 98 pg/g total lysate protein[21]	196	75	100	—	0.25	0.94	Measured in pleural fluid cell lysates. Only tested in MPEs of adenocarcinomatous lineage. About 40% cytology-negative MPEs tested positive for EpCAM

AUC: area under the curve; CA: carbohydrate antigen; EpCAM: epithelial cell adhesion molecule; HE4: human epididymis secretory protein 4; LR: likelihood ratio; MPE: malignant pleural effusion; proGRP: pro-gastrin releasing peptide; SCLC: small-cell lung cancer.

Soluble protein biomarkers in mesothelioma

Mesothelioma is an infrequent tumor that is difficult to diagnose because of the low yield of pleural fluid cytology[25]. The main soluble biomarker evaluated for its ability to establish the diagnosis of mesothelioma is mesothelin or soluble mesothelin-related peptides (SMRP); terms that are used interchangeably. Mesothelin is a glycoprotein involved in cell-to-cell adhesion and is expressed on the surface of normal mesothelial cells. In a meta-analysis of 21 studies (2,482 patients), pleural fluid mesothelin showed a sensitivity of 73% and a specificity of 90% for labeling mesothelioma[26]. The area under the curve (AUC) for this marker was 0.83 in another meta-analysis of 19 studies[27]. There is heterogeneity in the reported cutoff value, but the

most commonly used is 20 nM. According to the British Thoracic Society guidelines, mesothelin testing should be considered in patients presenting with suspicious cytology who are not candidates for more invasive diagnostic procedures[28]. Other biomarkers, including fibulin-3 (AUC = 0.68) and hyaluronic acid (AUC = 0.78), have not demonstrated sufficient discriminatory ability to be considered clinically useful[27].

IMMUNOCYTOCHEMICAL MARKERS

Pleural fluid cytological examination involves utilizing the cell pellet obtained after centrifugation. This process entails preparing a smear using Papanicolaou or May–Grunwald Giemsa stains and creating a formalin-fixed and paraffin-embedded (FFPE) cell block that is stained with hematoxylin–eosin[29]. Cell blocks, which closely resemble histological tissue, typically serve as the substrate for immunocytochemical analyses. It is important to note that smears and cell blocks are not mutually exclusive techniques; rather, they are complementary approaches that should be employed together in the examination of pleural fluid. For instance, in a study of 414 patients with MPEs, 11% of the samples identified as negative through cytological smears contained malignant cells on cell blocks[30]. Conversely, 15% of negative cell blocks were reported as positive for malignant cells on the smear slides.

In the initial evaluation of a pleural effusion, the pathologist assesses the morphological features of the cells to determine whether they are mesothelial or epithelial in origin and whether they are benign or malignant. The primary purposes of analyzing immunocytochemical biomarkers in pleural fluid cell blocks are (1) distinguishing mesothelioma from adenocarcinoma, (2) differentiating reactive from malignant mesothelial cell proliferation, (3) identifying the primary tumor that invades the pleural surface, and (4) predicting the effectiveness of immunotherapy or targeted therapies.

The choice of immunocytochemical markers among pathologists and institutions is influenced by several factors, including the preferences of individual pathologists and the performance of such markers in their respective laboratories.

Markers used to differentiate mesothelial from epithelial proliferations

Determining the pathological substrate of a pleural effusion requires distinguishing between reactive mesothelial proliferation, malignant mesothelial proliferation (mesothelioma), and epithelial proliferation (carcinoma) due to their overlapping cytomorphological features. This distinction is achieved through the use of immunocytochemical markers, the primary purpose of which is to identify the cellular lineage of suspected MPE as either mesothelial or epithelial.

In some centers, broad-spectrum cytokeratin staining (e.g., CKAE1/AE3 and CAM5.2) is the first diagnostic step. Cytokeratin cocktails are effective for screening epithelial

differentiation and epithelial neoplasms. Nevertheless, pan-cytokeratin markers are also expressed in virtually all benign and malignant mesothelial proliferations. Therefore, a negative result shifts the potential focus of the disease to tumor types such as lymphoma, melanoma, or sarcoma. To determine the mesothelial or epithelial lineage, it is recommended to employ a first-line immunopanel that incorporates two epithelial and two mesothelial markers, given that no single marker is infallible in terms of sensitivity and specificity. If the results are concordant, the diagnosis can be deemed conclusive. However, if the results are discordant, the immunopanel should be broadened for further rounds of immunostaining. In practice, it is common for samples to require processing with more than four stains because of contradictory staining results or the challenge of achieving the rule of two positive markers for one cell lineage and two negative markers for another.

The most reliable markers of epithelial proliferation are claudin-4, EpCAM (also referred to as Ber-EP4, MOC31, or CD326), and CEA (Table 3)[29,31,32]. In contrast, immunoreactive markers frequently used for mesothelial cells include calretinin, Wilms tumor 1 (WT1), D2-40/podoplanin, cytokeratin 5/6 (CK5/6), and heart development protein with EGF-like domains 1 (HEG1). Although there is no universally accepted criterion for the minimum percentage of tumor cell staining required to be considered "positive," employing a threshold of 10% appears to be a reasonable starting point[32].

In a meta-analysis of 14 studies, claudin-4 immunocytochemistry on cell blocks exhibited a pooled sensitivity of 98% and specificity of 99.7% for differentiating metastatic carcinomas from mesothelial cell proliferations (mesothelioma or reactive mesothelial cells)[33]. Researchers have proposed that this single-shot marker for carcinoma, when combined with a recently identified mesothelioma marker, HEG1 (clone SKM9-2), could accurately separate epithelioid/biphasic mesothelioma and nonsmall cell lung cancer (NSCLC) using only two immunostains[36]. However, HEG1 is not yet widely used in clinical practice. Furthermore, caution should be exercised when differentiating between mesothelioma and ovarian carcinoma, as all mesothelioma markers may stain ovarian carcinomas.

Markers used to differentiate benign from malignant mesothelial proliferations

Table 4 illustrates various markers that are typically utilized to confirm the mesothelial origin of cells in pleural fluid specimens and their accuracy in distinguishing pleural mesothelioma from benign mesothelial conditions. Markers with >90% sensitivity for mesothelioma include epithelial membrane antigen (EMA, clone 29), desmin loss, and HEG1[32,37]. The most specific markers for mesothelioma include loss of nuclear BRCA1-associated protein 1 (BAP1) and loss of cytoplasmic methylthioadenosine phosphorylase (MTAP). MTAP, tested by immunocytochemistry, is a reliable surrogate for cyclin-dependent kinase inhibitor A (CDKN2A) homozygous deletion (also

Table 3. Immunocytochemical markers for identifying carcinoma in general and lung carcinoma in particular[32-35]

Marker	Comments
Carcinoma markers	
Claudin-4	Pancarcinoma marker expressed as a punctate or continuous membranous staining. Constantly negative in mesothelial cells, mesothelioma, and cholangiocarcinoma.
EpCAM (clones MOC31 and Ber-EP4)*	Positive in >90% of lung adenocarcinomas and lung squamous cell carcinomas. Usually negative in benign and malignant mesothelial cells, but approximately 10–15% of epithelioid mesotheliomas may express focal positivity.
CEA	Strong membranous staining pattern in >85% of lung adenocarcinomas and lung squamous cell carcinomas. Carcinomas of various origins are usually negative (e.g., endometrioid, non-mucinous ovarian, prostate). Less than 5% of epithelioid mesotheliomas may show focal positivity.
Markers of lung carcinoma	
TTF-1	Nuclear staining in 70–80% of lung adenocarcinomas and small cell lung cancers. Mesotheliomas are negative. Metastatic adenocarcinoma to the lung is nearly always negative for TTF-1, except in metastatic thyroid malignancies.
Napsin A	The expression of napsin A and TTF1 is highly correlated. Positive cytoplasmic staining in 70–80% of lung adenocarcinomas. Mesotheliomas virtually always negative.
p40 or p63	Diffuse nuclear staining in >95% of lung squamous carcinomas. p40 is more specific than p63 for squamous cell carcinoma (p63 can be expressed in up to 30% of lung adenocarcinomas). About 5% of epithelioid mesotheliomas are positive for p40/p63.
INSM-1, chromogranin A, synaptophysin, CD56 (NCAM)	Markers of neuroendocrine tumors (e.g., small cell lung cancer). INSM-1 is the most accurate one (95% sensitivity and specificity). Synaptophysin is more sensitive than chromogranin A (90 vs. 50%).

*MOC31 and Ber-EP4 are monoclonal antibodies that bind to different epitopes on EpCAM.
CEA: carcinoembryonic antigen; EpCAM: epithelial cell adhesion molecule; INSM1: insulinoma-associated protein 1; NCAM: neural cell adhesion molecule; TTF-1: thyroid transcription factor 1.

referred to as the loss of p16 nuclear expression) detected by fluorescence *in situ* hybridization (FISH). Although not present in all cases of mesothelioma, loss of nuclear BAP1 by immunocytochemistry, loss of MTAP by immunocytochemistry, and homozygous deletion of CDKN2A by FISH are characteristics not found in benign mesothelium. Whereas BAP1 loss is more sensitive for epithelioid mesothelioma than biphasic/sarcomatoid mesothelioma, the opposite is true for MTAP loss and CDKN2A homozygous deletion.

In a study comprising 59 paired samples of pleural biopsy and pleural effusion cell blocks from mesothelioma patients, there was a significant degree of concordance in immunocytochemical staining for various markers, including EMA, calretinin, D2-40/podoplanin, CK5/6, WT1, desmin, and BAP1, with a range of 86–98%[38]. However, the concordance between cytology and histology specimens was lower for MTAP (72%), and particularly for the sarcomatoid histological subtype, with a concordance of <45% for all markers.

Table 4. Immunocytochemical markers for mesothelial cells (malignant or benign)[26,31,32,37]

Marker	Sensitivity (%)	Specificity (%)	Comments
	For mesothelioma vs. reactive mesothelium[a]		
Calretinin	75	64	Only nuclear staining is interpreted as positive. Sensitivity of 94% for epithelial mesothelioma, but just 50% for sarcomatoid subtype. Can be positive in 20–60% of adenocarcinomas.
WT1	69	0	Only nuclear staining is diagnostic. Sensitivity of 85% for epithelial mesothelioma. Also expressed in 80–90% of ovarian malignancies and 20% of other metastatic adenocarcinomas.
D2-40/ podoplanin	80	10–20	Strong membranous staining is considered positive. Weak or focal membranous staining is seen in 10–60% of ovarian adenocarcinomas. Podoplanin may be positive in half of squamous cell carcinomas.
HEG1	89	35	Sensitivity of 97% for epithelial mesothelioma. Membranous HEG1 expression may be seen in ovarian adenocarcinomas (65%), but not in lung adenocarcinomas.
GLUT1	70–80	90–95	Expressed in a variety of malignancies (head and neck squamous cell carcinoma, pancreatic cancer, endometrial cancer, mesenchymal tumors)
IMP3	65	95–100	Expressed in a variety of malignancies (renal cell carcinoma, pancreatic adenocarcinoma, chondrosarcoma, melanoma).
GATA3	53	100	More sensitive for sarcomatoid (65%) than epithelial (37%) mesotheliomas. Expressed in >90% of breast carcinomas and >80% of urothelial carcinomas; less frequently in squamous cell carcinomas.
CK5/6	58	100	Immunoreactivity to CK5/6 is seen in virtually all squamous cell carcinomas and a significant percentage of adenocarcinomas.
CKAE1/AE3	92	0	This stain does not differentiate benign from malignant mesothelial proliferations. Positive reactions are expected in epithelial cells, including normal and malignant cells.
EMA (clone E29)	95	90	Expressed in adenocarcinomas (>90%). Diffuse cytoplasmatic staining pattern in carcinomas, and cytoplasmic membrane staining pattern in mesotheliomas.
Desmin loss	90	70	Benign mesothelial cells tend to express desmin and lose their cytoplasmic desmin expression as they transition to malignancy.
Mesothelin	65–70	20–30	Expressed in 95% of serous carcinoma of the ovary, and more than half of endometrial carcinomas and lung adenocarcinomas.
BAP1 loss	65	99	Only nuclear loss of staining is accepted as true loss of expression. BAP1 loss is seen in just 25% of sarcomatoid mesotheliomas. BAP1 loss may also be encountered in malignancies such as renal carcinoma, cholangiocarcinoma, and melanoma (15%), but in <1% of lung and ovarian cancers.
MTAP loss	50	99	Only cytoplasmic loss should be interpreted as true loss of expression. Loss of MTAP may also be seen in approximately 75% of sarcomatoid mesotheliomas, and 20% of urothelial, biliopancreatic, and gastroesophageal cancers.

[a]Typically, a minimum of 10% tumor cell staining is necessary to be considered "positive staining".

BAP1, BRCA1-associated protein 1; CK, cytokeratin; EMA, epithelial membrane antigen; GATA3, GATA binding protein 3; GLUT1, glucose transporter 1; HEG1, heart development protein with EGF-like domains 1; IMP3, insulin-like growth factor II mRNA-binding protein 3; MTAP, methylthioadenosine phosphorylase; WT1, Wilms tumor 1.

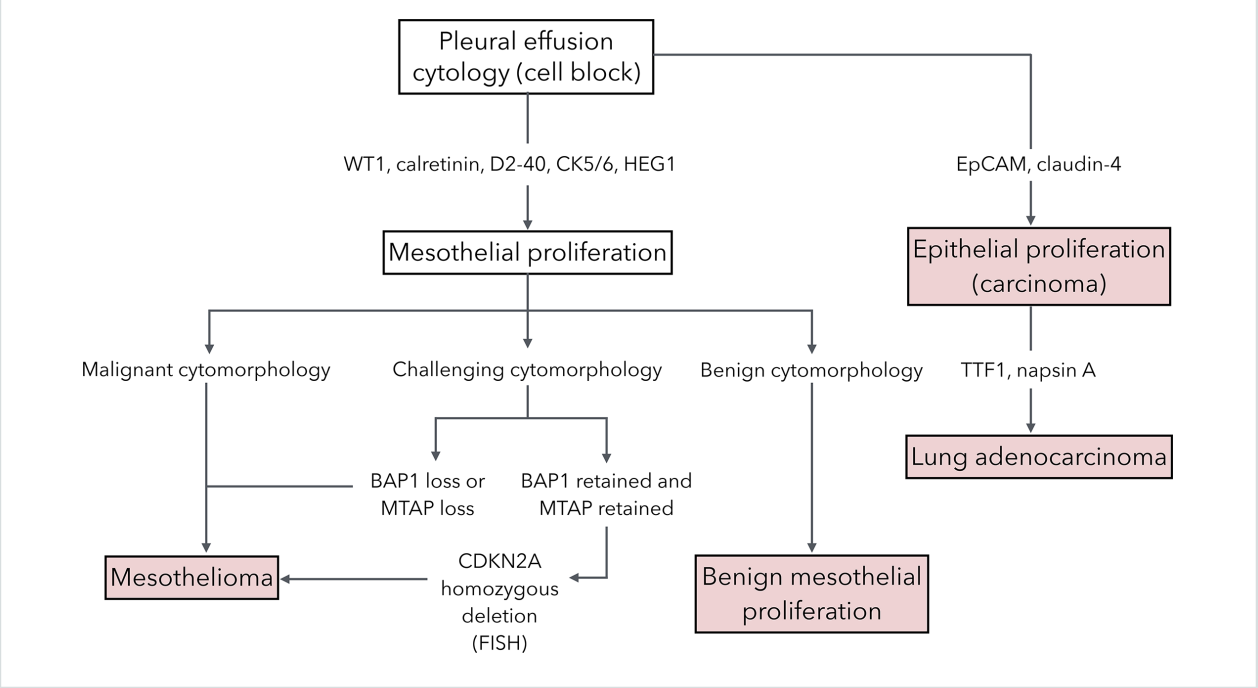

Figure 1. Algorithm for distinguishing between mesothelial and epithelial proliferation in pleural fluid cell blocks. BAP1: BRCA1-associated protein 1; CDKN2A: cyclin-dependent kinase inhibitor A; CK: cytokeratin; EpCAM: epithelial cell adhesion molecule; FISH: fluorescence in situ hybridization; HEG1: heart development protein with EGF-like domains 1; MTAP: methylthioadenosine phosphorylase; TTF-1: thyroid transcription factor 1; WT1: Wilms tumor 1.

The combination of BAP1 and MTAP, where loss of staining for either marker is considered positive, detects approximately 73–84% of mesotheliomas, with a specificity ranging from 85% to 100%[39,40]. Currently, optimal performance is achieved through the combination of BAP1 loss assessed via immunohistochemistry and CDKN2A evaluated using FISH, which yields a sensitivity of 81–83% and a specificity of 95–100%[26,40]. However, due to the greater technical complexity of FISH and its lack of availability in many centers, it is reserved for cases in which malignant mesothelial proliferation is suspected despite the presence of retained BAP1 and MTAP (Fig. 1).

Markers used to establish the primary tumor type in malignant effusions

In our unpublished experience with more than 1,000 cases of MPE, the primary tumors were lung (43%), gastrointestinal (13%), breast (11%), hematological (10%), gynecological (8%), unknown origin (6%), renal (3%), and mesothelioma (2%). Although some studies have reported that approximately 10% of MPE cases have an unknown primary tumor type[41,42], recent advances in immunohistochemical staining have halved this figure. Table 5 provides a concise summary of the recommended immunostains that can help to identify the potential origin

Table 5. Tumor-specific immunocytochemical markers*

Tumor origin	Markers	Comments
Lymphoproliferative neoplasms	CD45, CD19, CD20, CD10, HHV-8	These markers are more commonly assessed by flow cytometry. CD45 is a marker for hematopoietic and lymphatic tumors. CD19 and CD20 are pan-B lymphocyte markers. HHV-8 is a marker of primary effusion lymphoma and other related lymphoproliferations.
Breast	GATA-3, estrogen and progesterone receptors, mammaglobin, GCDFP15	GATA-3 is found in up to 90% of breast carcinomas in females, except in triple-negative cases (<70%). Mammaglobin is expressed in 60–80% of cases, and the remaining markers in approximately 60%.
Gynecological tract	Estrogen receptors, PAX-8, p16 (CDKN2A), WT1	Estrogen receptors and PAX-8 are expressed in endometrioid carcinoma, whereas PAX8, WT1, and p16 stain serous ovarian carcinoma.
Pancreaticobiliary and upper gastrointestinal	CK7, CK20, CK19, PDX-1, CDX-2, villin	CK7+/CK20– (majority of pancreas and gastric), CK7+/CK20+ (majority of biliary); CK19, villin, and CDX-2 are expressed in gastric adenocarcinoma.
Liver	Hep Par-1, CD34, Glypican-3, AFP	Due to formalin fixation and tissue processing, up to 50% of hepatocellular carcinoma turns negative for AFP in immunocytochemistry.
Colorectal	CK7, CK20, CDX-2, SATB-2, PDX-1, villin	CK7–/CK20+ (66%) in combination with CDX-2+ is highly suggestive of colorectal cancer.
Renal	PAX-8, RCC, CA IX, CD10	CK7±/CK20–. Napsin A is expressed in 80% of papillary renal cell carcinomas and rarely in clear cell ones.
Urothelial	CK7, CK20, GATA-3, uroplakins, p63	CK7+/CK20+ (60%), CK7+/CK20– (30%). p63 is not specific for urothelium and is positive in squamous tumors from other sites.
Prostate	PSA, NKX3.1, prostein	NKX3.1 has a high sensitivity and specificity but can be positive in male breast cancer. 10% of high-grade prostatic carcinomas are negative for PSA.

*For markers of lung tumors, see Table 3.

AFP: α fetoprotein; CDKN2A: cyclin-dependent kinase inhibitor A; CDX-2: caudal-related homeobox 2; CK: cytokeratin; CK: cytokeratin; GATA-3: GATA binding protein 3; GCDFP15: gross cystic disease fluid protein 15; HHV-8: human herpervirus 8; CA IX: carbonic anhydrase 9; NKX3.1: NK3 homeobox 1; Hep Par-1: hepatocyte paraffin 1; PAX-8: paired box 8; PDX-1: pancreatic and duodenal homeobox 1; PSA: prostate-specific antigen; RCC: renal cell carcinoma marker; SATB-2: special AT-rich sequence-binding protein 2; WT1: Wilms tumor 1.

of MPEs. A relatively extensive panel of antibodies may be necessary because there are no antibodies exclusively specific to a particular tumor type.

Markers used for indicating immunotherapy

Programmed death ligand 1 (PD-L1) is an immune checkpoint protein that can be expressed on tumor cells and regulates the antitumor immune response by inhibiting T-cell-mediated cell death. Detection of PD-L1 in pleural fluid cell blocks can be used to predict the clinical response to PD-1 or PD-L1 selective checkpoint inhibitors in patients with lung adenocarcinoma and other malignancies (e.g., head and neck squamous cell carcinoma, melanoma, triple-negative breast carcinoma, urothelial cancer, and gastric or esophageal cancer). Although eligibility for

immunotherapy generally requires immunocytochemical assessment of PD-L1, this is not always the rule (e.g., first-line therapy with certain immune checkpoint inhibitors in NSCLC, such as cemiplimab-rwlc monotherapy or atezolizumab with or without chemotherapy).

FFPE cell blocks derived from pleural fluid are considered the most suitable specimen type for PD-L1 analysis because they closely resemble biopsy samples[43]. A minimum of 100 viable tumor cells are required for PD-L1 evaluation[44]. The definition of positive or negative testing depends on the individual antibody, clone, and platform, which may be unique to each checkpoint inhibitor. For example, the interpretation of PD-L1 immunocytochemistry in NSCLC focuses on the proportion of tumor cells expressing complete or partial membrane staining: <1, 1–49, and 50% or more. First-line therapy for advanced or metastatic disease varies according to these percentages or tumor proportion scores.

Although immunocytochemistry can be used to screen for epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK) rearrangements, C-ROS oncogene 1 (ROS1) rearrangements, v-raf murine sarcoma viral oncogene homolog B1 (BRAF) V600E, Kirsten rat sarcoma virus (KRAS), human epidermal growth factor receptor 2 (HER2), and rearranged during transfection gene (RET) rearrangements, these markers are typically assessed and confirmed using molecular testing methods (see below)

FLOW CYTOMETRY MARKERS

Flow cytometry uses fluorescently labeled antibodies that bind to specific antigens on the cell surface or inside the cells. It may complement immunocytochemistry or be more reliable in detecting certain markers, allows for simultaneous detection of multiple markers, and has a rapid turnaround time (hours).

Flow cytometry in hematological malignancies

In current practice, flow cytometry supplements cytology to identify lymphomatous cells in pleural fluid. The sensitivities of cytology and flow cytometry for identifying malignancy in a small series of 27 patients with pleural effusion secondary to diffuse large B-cell lymphoma were comparable, at approximately 70%[45]

Since T lymphocytes represent approximately 70–80% of the pleural fluid lymphocyte population in patients with nonmalignant and nonhematological malignant causes of pleural effusions, a pleural fluid T/B cell ratio of <1 suggests lymphoma or leukemic etiology (85% of non-Hodgkin lymphomas are B-cell-derived)[46]. Flow cytometry detects the presence of a clonal B-cell population (e.g., over-representation of either kappa- or lambda light-chain-bearing cells). For example, to characterize diffuse large B-cell lymphomas, cell surface marker analysis using flow cytometry with pleural fluid specimens usually includes CD45, CD3, CD5, CD19, CD10, CD20, and kappa/lambda.

Interpretation of the results should be done cautiously in unusual cases of traumatic thoracentesis where peripheral blood contaminates the pleural fluid, as one could erroneously conclude that there is a pleural extension of the hematologic malignancy in a patient with other potential causes of the effusion. The use of flow cytometry should be restricted to patients with a history of hematologic malignancy who develop pleural effusion, or when there is a high clinical suspicion of lymphoma or atypical cytomorphological data. Otherwise, the probability of diagnosing an unsuspected hematologic malignancy is very low[47].

Flow cytometry non-hematological malignancies

The potential utility of flow cytometry for phenotyping malignant epithelial cells in pleural effusions is less well known. However, the detection of EpCAM-positive cells indicates the presence of an epithelial malignancy. In a study involving 140 MPE samples from epithelial cell tumors, the sensitivity of conventional cytology was 57%, whereas the sensitivity of flow cytometry (EpCAM-positive cells) was 83.5%, resulting in a 26% increase in detection rate compared to conventional cytology[48]. Certainly, the application of flow cytometry in the diagnosis of nonhematologic malignancies can mitigate the limited sensitivity of cytology and provide results in a much shorter time. Nonetheless, for this method to gain greater acceptance, it is essential that pathology departments acquire flow cytometers, as hematologists who typically employ this technique may face challenges in providing their services to patients with nonhematologic malignancies.

MOLECULAR MARKERS

In the era of personalized medicine, merely identifying a pleural effusion as malignant is no longer adequate. Rather, it is essential to explore the presence of molecular or genetic alterations (driver or oncogenic mutations) that affect therapy selection. A paradigmatic example of this is NSCLC (Table 6).

Suitable pleural samples for molecular analyses

Tissue biopsies have traditionally been considered the standard material for molecular analyses. In a study of 183 patients with MPE, the pleural biopsy technique and the size of the pleural biopsy were independently associated with successful molecular marker analysis. Specifically, specimens obtained by local anesthetic thoracoscopy had a greater chance of successfully achieving a molecular profile than computed tomography- or ultrasound-guided pleural biopsies (95 vs. 86 vs. 77%, respectively)[49]. Nevertheless, although thoracoscopically obtained biopsy samples offer superior diagnostic yields, this comes at the cost of invasiveness and potential complications.

Pleural fluid specimens are often the only or most appropriate materials available and should be subjected to molecular testing. In fact, the concordance between pleural fluid

Table 6. Genotyping of NSCLC and corresponding targeted therapies*

Molecular alteration	First-line therapy
EGFR exon 19 deletion or exon 21 L858R mutation positive	Osimertinib
EGFR S768I, L861Q, and/or G719X mutation positive	Afatinib or osimertinib
EGFR exon 20 insertion mutation positive	Amivantamab-vmjw (+carboplatin/pemetrexed)
KRAS G12C mutation positive	Sotorasib, adagrasib
ALK rearrangement positive	Alectinib, brigatinib, lorlatinib
ROS1 rearrangement positive	Entrectinib, crizotinib, repotrectinib
BRAF V600E mutation positive	Dabrafenib + trametinib, encorafenib + binimetinib
NTRK1/2/3 gene fusion positive	Larotrectinib, entrectinib, repotrectinib
METex14 skipping mutation positive	Capmatinib, tepotinib
RET rearrangement positive	Selpercatinib, pralsetinib
ERBB2 (HER2) mutation positive	Fam-trastuzumab deruxtecan-nxki
High-level MET amplification†	Capmatinib, tepotinib, crizotinib

*In addition to these molecular alterations, PD-L1 evaluated by immunocytochemistry can be used to identify tumors that are most likely to respond to first-line anti-PD-1/PD-L1.

†This is an emergent biomarker.

ALK: anaplastic lymphoma kinase; BRAF: v-raf murine sarcoma viral oncogene homolog B1; EGFR: epidermal growth factor receptor; HER2: human epidermal growth factor receptor 2; KRAS: Kirsten rat sarcoma virus; MET: mesenchymal–epithelial transition factor; NTRK: neurotrophic tropomyosin receptor kinase; RET: rearranged during transfection; ROS1: C-ROS oncogene 1.

and tissue for diverse molecular alterations is very high (>85%)[50]. FFPE materials, such as cell blocks, are suitable for most molecular analyses[51]. However, cytopathological preparations that are not processed using this method may be acceptable in certain cases. Moreover, examination of biological fluids, such as plasma or pleural fluid, to uncover the presence of biomolecules (nucleic acids, circulating tumor cells, tumor-educated cells, and extracellular vesicles) that are persistently secreted by tumor cells is becoming increasingly prevalent in clinical practice. This is known as liquid biopsy and allows the use of peripheral blood or pleural fluid as surrogate samples, particularly in the setting of advanced or metastatic disease[52,53]. One study showed that the detection rate of oncogenic mutations in cell-free DNA isolated from the pleural fluid supernatants of patients with lung adenocarcinoma was comparable to that of tumor biopsies (90.7 vs. 88%), and higher than that of pleural fluid cell blocks (48.5%) and plasma (75%)[54]. Pleural fluid supernatants and biopsies agreed in nearly 90% of specimens that had positive results for mutations. Therefore, pleural fluid cell-free DNA can be prioritized for detecting oncogenic mutations.

Testing methodologies

The testing methodologies include next-generation sequencing (NGS), Sanger sequencing, real-time polymerase chain reaction (PCR), and FISH. NGS is a high-throughput methodology that enables the rapid sequencing of large quantities of DNA or RNA in biological samples. This represents a faster and more cost-effective means of sequencing than traditional Sanger sequencing. Sanger sequencing is the most tumor cell-demanding method because it requires samples with a minimum of 25–30% tumor cell content after enrichment (i.e., when utilizing this method, it is essential to employ tumor enrichment techniques, such as macro- and microdissection). The identification of genetic mutations in samples using NGS, real-time PCR, and digital PCR techniques

necessitates tumor cell percentages as low as 0.01–5, 1–5, and < 1%, respectively. FISH typically requires a tumor cell percentage of approximately 20% (or 50–100 tumor nuclei to be identified in FFPE sections). PCR is used to interrogate specific mutations targeted by the assay, whereas FISH is used in certain circumstances for gene amplification events. Among the preceding techniques, broad-based genomic testing approaches, typically performed using NGS, are preferred. If broad panel genes do not provide identifiable driver oncogenes, RNA-based NGS (if not already performed) should be considered to maximize the detection of fusion events.

CONCLUSIONS

The diagnosis and treatment of malignant pleural effusion is often a challenge that requires a multidisciplinary approach[55]. In terms of diagnosis, the aim is to establish with certainty the malignant nature of an effusion using the least invasive and most cost-effective procedures. This is where pleural fluid biomarkers become important[56]. Some biomarkers, such as soluble protein biomarkers, indicate a high probability of malignancy, which must be confirmed cytohistologically. Immunocytochemical biomarkers are used to determine the precise origin of the tumor invading the pleura. Finally, molecular biomarkers are used to phenotype tumors, thereby allowing the identification of targeted therapies. In this sense, liquid biopsy represents a major advancement in the evaluation of malignant pleural effusions.

ETHICAL DISCLOSURES

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article. Furthermore, they have acknowledged and followed the recommendations as per the SAGER guidelines depending on the type and nature of the study.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Use of artificial intelligence for generating text. The authors declare that they have not used any type of generative artificial intelligence for the writing of this manuscript, nor for the creation of images, graphics, tables, or their corresponding captions.

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