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Review Article

Detection of carcinoma in serous effusions: a review

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Abstract: A malignant serous effusion is one of the most common complications of advanced tumors, indicating a poor prognosis and having a profound impact on diagnosis, treatment, and prognosis. It is of great significance to identify benign and malignant effusions quickly and accurately. Both cellular and non-cellular components in the effusion can be employed for detection, diagnostic methods are necessary to obtain a definite diagnosis and more relevant information such as tumor classification. In this review, we focus on the comparison of several widespread cytological preparation methods, enrichment technology of exfoliated cells, and present tests for serous effusions, mainly including routine and special stains, immunocytochemistry, electron microscopy, enzyme-linked immunosorbent assay, flow cytometry, and molecular analysis.

Keywords: Serous effusions, cytological materials, preparation methods, cell enrichment procedures, molecular analysis

Introduction

Serous effusions include pleural, peritoneal, and pericardial effusions, which are the pathological accumulation of fluids in the body cavity caused by various benign or malignant diseases. A malignant serous effusion is one of the common complications of advanced tumors, indicating a poor prognosis and having a profound impact on systemic anti-tumor treatment and quality of life [1]. Therefore, it is critical to make a distinction quickly and accurately between benign and malignant effusions [2].

In clinical practice, effusion is a repeatable sample of tumor cells, sometimes even the only source of specimens available. The effusion is often removed for therapeutic purposes by minimally invasive operation. Many components can be detected including floating viable exfoliated cells, free proteins, nucleic acids and other molecular components in the serous fluids of patients with cancer [3]. The interference of numerous benign cells, such as reactive mesothelial cells or inflammatory cells, is troublesome. Metastatic adenocarcinoma, malignant mesothelioma, and reactive mesothelial cells show morphological overlaps, and a single morphological test has difficulty distinguishing among them [4-6]. Thus, it is necessary to apply ancillary methods for definite diagnosis and more relevant information such as tumor classification. Some markers are relatively organ-specific, giving clues to the origination of the tumor. Notably, the medical history, clinical features, and radiological findings should also be considered for a comprehensive analysis.

This review introduces the preparations of cytological materials, the enrichment process of interest exfoliated cells, and contemporary diagnostic methods. The diagnostic procedures of effusion specimens are showed in **Figure 1**. We comprehensively describe the basic steps, key details, latest developments, and pros and cons of each method, which helps researchers make an optimal choice.

Preparations of cytological samples

Many factors account for the diagnostic efficiency of serous effusions, such as study size (large or small-scale studies), sample volume and types, pretreatment procedures, ancillary tests, and experience of analysts [7-10]. Here, we show the most frequent preparation meth-

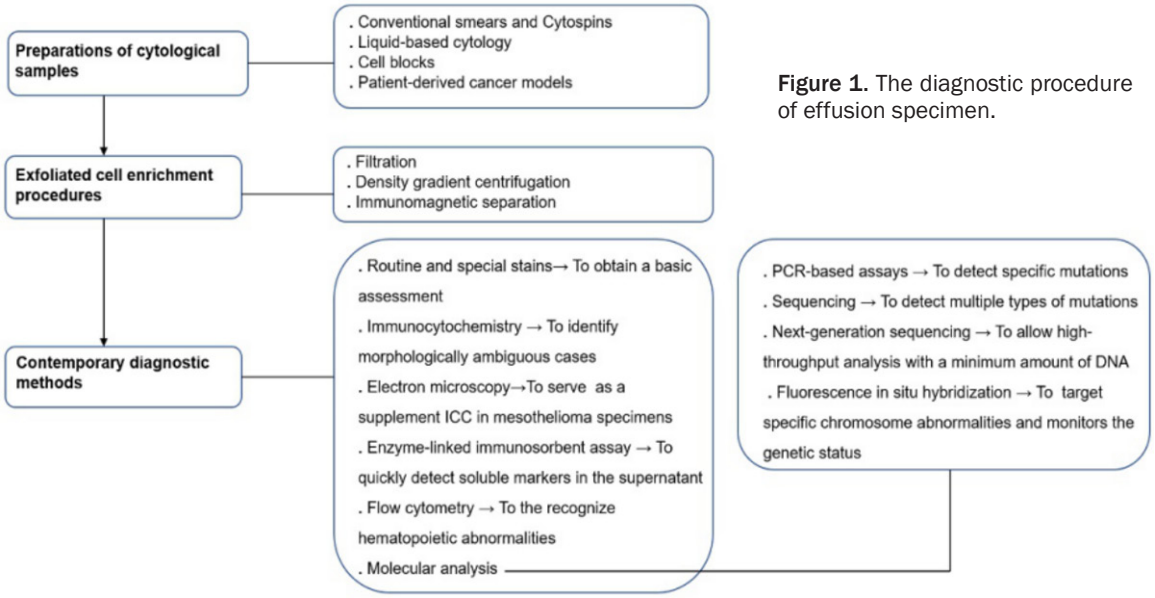

Figure 1. The diagnostic procedure of effusion specimen.

ods of serous effusions, including direct smears, cytospins, liquid-based preparations, cell blocks (CBs), and patient-derived cancer models [11-13]. Because of the limited number of malignant cells, the diagnosis of effusion is difficult. Some methods can be applied to enrich the interest cells to improve the diagnostic performance. We mainly introduce filtration, density gradient centrifugation, and immunomagnetic cell separation [14-19].

The volume of the specimens is related to the adequacy and validity of the cytological diagnosis. To improve diagnostic performance, the optimal minimum cutoff volume of peritoneal, pleural, and pericardial effusions is 200 mL, 65 mL, and 60 mL, respectively [20-22]. As the clotting of collected effusions may disturb diagnosis, heparin-pretreated needles or tubes are recommended for anticoagulation, especially in bloody samples, which do not affect the morphological characteristics of cells but reduce pH values [23, 24]. A fresh effusion is best for cytological tests or further study [25]. When the serous samples cannot be processed in time, they can be stored in a refrigerator at 4°C. The morphology and immunostaining patterns of the fluid may change slightly after 14 days, but they have no effect on the final diagnosis, and the fluid still retains sufficient DNA for molecular analysis [26].

The effusion can also be pre-fixed with an equal volume of 50% alcohol or methanol-based PreservCyt preservation solution, but the former fixation affects the staining forms of Diff-Quik staining and some immunocytochemistry (ICC) markers [8]. Notably, air-dried preps cannot be prepared once pre-fixed [25]. For hemorrhagic fluids, the lysate can be added to remove red blood cells, such as 1% glacial acetic acid or 0.15 M ammonium chloride solution [27, 28]. After collecting, the effusion is centrifuged, and the pellet is taken into the subsequent process. The remaining materials can be frozen in 10% Roswell Park Memorial Institute/dimethyl sulfoxide (RPMI/DMSO) medium at -80°C for further investigations [28]. The comparison between different cytological preparations is described in **Table 1** [13, 25, 29-38].

Conventional smears (CSs) and cytospins

CSs are made by spreading the centrifuged sediment onto glass slides. It is recommended to use a cotton swab instead of pipette-"hematologic" two-slide technic for better cellular aggregation and morphology [39]. Direct CS is the easiest, fastest, and most economical method [25]. The defects of CSs are (1) lower sensitivity due to cells overlapping, cell loss, and different laboratory procedures [34]; (2) relatively few and random materials are necessary to make smears, and the remaining discarded samples may contain important diagnostic information [29]; and (3) a large number of reactive mesothelial cells and inflammatory cells may disturb the observation of the detailed

Table 1. Comparison of different cytological preparation methods

Cytological preparations	Strength	Shortcoming	Ref
Conventional direct smear	The process is simple, fast and economical Cells remain intact, with high quality DNA and RNA	Lower sensitivity due to cells overlapping, cell loss and chaotic background Remaining discarded fluids may be informational The coverslip needs to be removed	[25, 29, 34]
Cytospins	The collected samples can be fully utilized Cells remain intact, with high quality DNA and RNA	The coverslip needs to be removed	[38, 41]
Liquid-based preparations	The background is clear Unstained slides can be used for other tests Cells are well preserved, with high quality DNA and RNA	The coverslip needs to be removed	[30, 31]
Cell blocks	Cellular details are well preserved Numerous sections facilitate multiple analysis and archival storage Serving as a bridge connect cytology and histology	Formalin fixation affects DNA quality The process is labor-intensive and time-consuming Limited application when there are few cells	[13, 29, 34-37]
Patient-derived cancer models	Biological and molecular characteristics of human tumors are accurately recapitulated The models can be applied to learn biological behaviors, choose treatment plans, observe drug responsiveness, and predict treatment effects	These procedures are time-consuming, expensive, and not available in every laboratory Success rate is limited, discrepant in different tumors	[12, 47-50]

morphology of atypical or malignant cells, which increases the difficulty of diagnosis. Therefore, CSs are often applied in combination with other techniques such as CBs, especially in a diagnostic dilemma [40].

Cytospins refer to the preparation of smears by using cytocentrifuges, also known as cytospin smears, which coat the resuspended pellets on a slide at a certain speed [41]. Compared to direct smear, cytospin, as an enrichment method, can reduce diagnostic traps associated with unskilled technique and make full use of the collected samples, which is a highly accurate, convenient alternative method [38]. Both the cytospins and CSs must be fixed before staining. The fixation method is related to the type of staining. Air-drying is required before Romanowsky techniques such as Giemsa stain, and 95% ethanol fixation is generally applied before Papanicolaou and Hematoxylin-Eosin (HE) stain [37, 42]. For immunocytochemical stains, 100% cold methanol is recommended to maintain the immunoreactivity of different antigens, such as hormone receptors, nuclear or lymphoid antigens [32, 33].

Liquid-based cytology (LBC)

ThinPrep is the preferred liquid-based preparation in many laboratories. The general steps for ThinPrep are as follows [43]: first, the collected sample is fixed with preservative solution CytoLyt, then centrifuged, and second, the precipitate is resuspended and put into the ThinPrep® Processor, following the manufacturer's instructions for automatic slide production. The remaining solutions can be stored for further research. The prepared slides are generally fixed with alcohol and analyzed by Papanicolaou stains; unstained slides can be air-dried and stored at -70°C for up to 1 week for immunocytochemical stains [11]. The advantages of the ThinPrep method are described as follows: (1) the cells are well preserved; (2) the overall background is clear because of reduced air-drying artifacts, blood, or inflammatory cells, which facilitates the observation of morphological details and the diagnosis of samples; (3) the additional unstained slides can be used for other auxiliary tests, such as ICC or molecular analysis [30, 31].

CBs

Although no standard method for the preparation of CBs is available, 3 major acceptable steps are cell concentration, formation of a sticky pellet, and histological treatment of the pellet [36]. Cell concentration means discarding the supernatant after centrifugation. Plasma thrombin clot and the HistoGel technique have been widely adopted to form a sticky pellet by binding cells together with some viscous media. The former is to add 2-3 drops of plasma and thrombin to the precipitate to form a clot; the latter is to mix HistoGel heated into liquid with the sediment and solidify at room temperature. The remaining materials of ThinPrep stored in CytoLyt solution can also be processed by the plasma thrombin method [44, 45]. There are many methods for CBs fixation, different fixation methods should be selected for corresponding auxiliary analysis [36]. Formalin fixation is a relatively conventional method. Subsequently, immunohistochemical staining and molecular tests can be performed. These tests can also be applied after alcohol fixation. Because formalin may cause chemical cross-links of DNA fragments affecting analysis, alcohol or a mixture of both are chosen for sample fixation [46]. The formalin-fixed paraffin-embedded (FFPE)-CB is usually cut to 4-6 μm, and its routine staining is HE.

The merits of CBs are described as follows [13, 29, 34, 37]: (1) the architectural patterns are well preserved, such as cell balls, papillae, three-dimensional clusters, connections between cells, cytoplasmic, and nuclear morphology, which makes it convenient to interpret the staining forms and improves the diagnostic sensitivity; (2) a single sample can produce numerous sections facilitating archival storage for further research, and these sections can be tested simultaneously for multiple forms of staining or molecular analysis; (3) sufficient cells can be obtained and gathered in a small area for easy microscopic observation, serving as a bridge connecting cytology and histology. The shortcomings of CBs are as follows: highly technical, labor-intensive, time-consuming, and limited application when there are few cells [35, 36].

The selection of cytological material preparations is related to, for example, sample types, estimated diagnosis, and laboratory preference [36]. CB is recommended as an adjunctive preparation for smear or LBC, especially when the diagnoses of both are negative or difficult to define, and their remaining fluids are

usually exploited to prepare the CBs [34, 37, 40].

Patient-derived cancer models

Pre-clinical models include cancer cell lines, patient-derived xenograft (PDX), spheroid culture and patient-derived organoid (PDO), etc [12, 47]. Among them, patient-derived cancer models gain extensive attention due to their accurate recapitulation of the biological and molecular characteristics of human tumors in vivo. Scholars isolated tumor cells from malignant effusions, cultured them in vitro to form PDO models, or injected them into immunodeficient mice to obtain PDX models [48, 49]. Molecular analysis of effusion is a valuable tool providing useful information for precision medicine. Pre-clinical tumor models and molecular analysis are combined to learn biological behaviors, choose treatment plans, observe drug responsiveness, and predict treatment effects [12, 50]. The disadvantages of these models are: (1) these models are time-consuming, expensive, and not available in every laboratory; (2) the success rate is limited, discrepant in different tumors.

Sarah J Hill et al. built a PDO model using pleural effusions to learn DNA damage repair and test therapeutic sensitivities for a rapid targeted drug screening in patients with high-grade serous ovarian cancer (HGSOC) [49]. Giuseppe Roscilli et al. established a PDO model using malignant pleural effusions to study the heterogeneity and predict chemosensitivity in patients with advanced non-small cell lung cancer (NSCLC) [51]. Benjamin Izar et al. generated a PDX model with malignant ascites [48]. They exploited single-cell RNA sequencing to identify inter- and intra-patient heterogeneity and assess the anti-tumor activity of inhibiting the JAK/STAT pathway in HGSOC. Akihito Machinaga et al. established a PDX model to test the efficacy of gemcitabine and clarify the potential mechanism of chemotherapy resistance in patients with refractory pancreatic ductal adenocarcinoma [52].

Exfoliated cell enrichment procedures

Many other exfoliated cells in the serous effusions interfere with the detection of malignant cells. As shown by Bertrum Sheid et al., ascites in patients with ovarian cancer contained only <0.1% of adenocarcinoma cells, 37% of lymphocytes, 32% of macrophages, and 29% of mesothelial cells [53]. Fortunately, there are slight differences in the size, density, and expression of surface biomarkers between malignant and normal cell groups, which can be utilized to isolate and enrich a sufficient number of tumor cells for analysis. The main enrichment measures are filtration, density gradient centrifugation, and immunomagnetic separation [15, 18, 19, 54-62] (**Table 2**).

Filtration

Filtration is a simple, fast, and cost-effective separation method based on cell size but has limited applications because the separation result is not as good as that for the latter 2 procedures. More than half of the tumor cells exist in clusters, whereas most non-malignant cells exist as a single cell, and the tumor cells derived from epithelium are usually larger than leukocytes [18, 55]. Therefore, filtration membranes can be utilized for filtration. The pore size of the filtration membrane is the key factor, and it should be selected according to the different target cells. H. W. HIRTE et al. used a 30 μm nylon mesh filter to isolate ovarian cancer cells from ascites [18]. Elin Andersson et al. applied an 8 μm commercial track-etched polycarbonate filter to isolate bladder cancer cells from urine [55]. This approach is relatively inaccurate with many disadvantages. The malignant cells defined in the filtration procedure are clumps of cells, which is controversial because not all tumor cells exist in the form of aggregation [57].

Density gradient centrifugation

Density gradient centrifugation is a physical separation method based on the density divergence between different cells. It is applied to separate specific cells in serous effusions or peripheral blood. Density gradient media are, for example, Percoll, BSA, Ficoll, and Renografin [19]. Percoll is the comparatively favored medium among them because it is commercially available, with relatively stable physical and chemical properties, simple density gradient adjustment, easy elution, and gentle damage to cells [19, 54].

One example of the operation flow is as follows: a discontinuous Percoll density gradient

Table 2. Comparison of different cell enrichment procedures

Enrichment procedures	Basic principles	Strength	Shortcoming	Ref
Filtration	Divergence of cell size	Simple, fast, and cost-effective	Low purity and cell yield	[15, 18, 57]
Density gradient centrifugation	Divergence of cell density	Simple, fast, more accurate than filtration	Low purity and cell yield	[15, 19, 54, 58]
Immunomagnetic separation	Specific binding of antibodies to different cellular markers	Higher yield and purity of interest cells Especially suitable for sorting rare cells Commercially available chips for continuous high-throughput analysis	Time-consuming and expensive Mostly applied for laboratory analysis	[15, 56, 60, 62, 71, 52-56]

of 60%, 50%, and 40% was prepared and placed in a centrifuge tube layer by layer; samples were collected for centrifugation and the precipitate was resuspended; cells were adjusted to a proper concentration (e.g., 10^7) and then layered on top of the gradient in 1 ml of 30% Percoll to centrifuge for 30 min at 800 g; finally, the cells of each layer were collected, eluted, and prepared for smears and staining tests. Approximately 90% of recovered malignant cells were found in the lowest density fraction, that is, less than 1.056 g/ml (corresponding to 30% Percoll gradient); 82% macrophages at a density of 1.056-1.067 g/ml (30%-50%); and 98% lymphocytes at 1.067-1.077 g/ml (40%-60%) [19]. Jerzy Rabczynski et al. pointed out that typical malignant ovarian cancer cells are concentrated in the density layer of 1.035-1.070 g/ml [63].

The loss of many target cells in the density gradient is due to the obvious difference in the density of tumor cells. This method can be combined with magnetic cell separation because it can effectively remove cellular debris and red blood cells, so as not to block the magnetic beads [58].

Immunomagnetic separation

The 2 key factors to assess the effectiveness of cell purification methods are yield and purity. Although the 2 former methods can obtain a large yield of cells, the purity is poor and the volumes of required effusions are large, and the immunomagnetic separation provides both higher yield and purity [15]. The general steps of immunomagnetic separation are as follows: incubate antibody-conjugated magnetic beads with collected effusions, and antibodies with high affinity for the specific surface antigen of the target cells connect the magnetic beads to the interest cells [58]; next, the mixed solution passes through a magnetic column, and the labeled cells are left to be eluted if necessary, with the unlabeled cells out [61].

According to the different targeting of the selected antibody, it is divided into positive and negative selection; the former antibody targets the desired interest cells, and the latter antibody targets the unwanted cells [64-66]. For example, antibodies exploited for positive separation of ovarian cancer cells include the following [14, 15, 28, 67-69]: Ber-EP4, folic acid, human epithelial antigen, the extracellular domain of the MUC16 cell surface protein, carbohydrate antigen 125 (CA125), a receptor tyrosine kinase, EphA2, and monoclonal antibody (mAb) CC49. Although CD45 is exclusively expressed on hematopoietic cells, it is routinely applied in negative separation to remove contaminating leukocytes [70].

The use of positive sorting is limited in the absence of phenotype information on the target cells. Some researchers hope that the enrichment step does not directly modify or connect the target cells, although the binding of the antibody with magnetic particles does not affect cell function [71]. The 2 methods should be selected according to the actual situation, and sometimes they can be exploited in combination [28, 65]. The cells remain intact and viable after magnetic isolation and could be tested for further morphological or immunocytochemical assays [56].

Immunomagnetic separation is employed in sorting the rare cells, which can achieve approximately 1000 times the enrichment of the initial samples, but it is time-consuming and expensive [56]. Commercially available chips have been introduced by some laboratories to support continuous high-throughput analysis and they are convenient, cost-effective, and promising for future applications [60, 62].

Routine and special stains

Routine and special stains are important diagnostic techniques for effusion cytology, especially cytochemistry and ICC. All cytological preparations can be employed for staining; among them, the CB is optimal with sufficient material [35, 37, 40]. Giemsa staining is a conventional way to air-dry smears; Papanicolaou or HE staining is a traditional method for alcohol-fixed smears; HE staining is generally performed in CBs [13, 37, 42]. Some special stains can be adopted to identify pathogens in morphologically suspicious infected samples, such as the Grocott or PAS stain to identify fungi, and the acid-fast or Fite stain to recognize mycobacterium [72].

When evaluating and interpreting the slides, researchers have focused on the cellularity, cell arrangement, and cytoplasmic and nuclear details, as comprehensively illustrated by Mair

et al. [34, 40, 73]: (1) volume of blood/clot obscuring background (large: 0, moderate: 1, minimal: 2); (2) amount of diagnostic cellular material present (minimal: 0, moderate: 1, abundant: 2); (3) degree of cellular degeneration and cellular trauma (marked: 0, moderate: 1, minimal: 2); and (4) retained architecture/cellular arrangement (minimal: 0, moderate: 1, excellent: 2). According to the aforementioned criteria, the quality of the slides is classified into 3 categories [34, 40, 73]: (1) diagnostically unsuitable (0 score); (2) diagnostically adequate (1-4 score); and (3) diagnostically superior (4-8 score). After the assessment, the final pathological diagnoses are usually divided into the following 5 groups [20, 74, 75]: benign, malignant, suspicious (more likely to be malignant), atypical (not completely consistent with benign or malignant cells in general morphology), and non-diagnostic (insufficient cells number or contaminated background).

ICC

The distinction between benign and malignant effusions is essential for diagnosis, treatment, and prognosis [76]. Cytochemistry staining is based on cellular morphology, and it is difficult to distinguish between benign and malignant cells in many cases, especially in metastatic adenocarcinoma, reactive mesothelial cells, and malignant mesothelioma. Because reactive mesothelial cells are hyperplastic and hypertrophic, closely mimicking malignant cells, metastatic carcinoma and malignant mesothelial cells exhibit morphological overlap, and a single cytological examination is insufficient for accurate diagnosis [4-6]. ICC is a valuable tool for solving this problem [76-78]. The FFPE-CB is the preferred preparation because of its clear background, multiple archival sections, and comparable performance to those of surgical pathological materials [35, 37].

Due to the heterogeneous expressions of tumor antigens, the diagnostic performance of a single antibody is limited, and a panel of markers is recommended for detection [76, 79]. No consensus on the best antibody combination has been reached. It should contain at least 2 antibodies, an epithelial marker, and a mesothelial one [8, 80]. Among them, MOC-31 is one of the most sensitive, specific adenocarcinoma markers that recognizes the epithelial cell adhesion molecule (Ep-CAM) on the surface of epithelial cells [78]. Calretinin is considered a reliable marker of mesothelioma [81]. Epithelial membrane antigen (EMA) is strongly positive in almost all mesothelioma and only weakly positive in reactive mesothelial cells, but desmin is the opposite [82]. The combination of BerEp4/Calretinin, desmin/EMA, or WT1/AE1-AE3 shows satisfactory performance, for example, EMA positivity and desmin negativity were found in 98% (49 of 52) of malignant mesothelioma, and EMA negativity and desmin positivity were revealed in 86% (55 of 64) of reactive mesothelial hyperplasia [82, 83]. Some markers can indicate the primary origin of metastatic cancer, such as Thyroid transcription factor-1 (TTF-1) to label pulmonary adenocarcinoma, CDX2 to mark intestinal adenocarcinomas, and GATA3 to suggest metastatic breast carcinoma [84-86].

Unlike immunohistochemical staining of surgical specimens, there remains no accurate immunocytochemical grading and scoring system for effusion. The immunostaining of some effusion samples is evaluated by semi-quantitative scoring, as has been described by Vickie Y et al. and Tomohiro Oda et al. [87, 88]. A staining index is recorded as the sum of the intensity score (IS) and percentage score (PS). IS refers to the staining intensity of the target cells in the corresponding expression pattern (core 0: no staining; 1: weak; 2: moderate; 3: strong), and PS refers to the proportion of positively stained interest cells (score 0: no staining; 1: <10%; 2: 10%-50%; 3: >50%). Some inspiration may be from the calculation of the M-score, an immunohistochemical scoring algorithm that also includes the proportion and staining intensity of positive cells [89, 90].

Electron microscopy (EM)

EM is applied to evaluate cellular ultrastructure, based on the morphology of microvilli, to identify mesothelial or epithelial cells [91]. The basic procedure is to fix the precipitate with glutaraldehyde after centrifugation, and then slice and embed it for EM evaluation [91]. Traditionally, EM has been the gold standard for the diagnosis of mesothelioma, but it is gradually being replaced by IHC because of its long time, high cost, and technical complexity [72, 92]. Now, the EM is only applied as an alternative method when the ICC diagnoses are ambiguous or difficult to interpret [93].

Enzyme-linked immunosorbent assay (ELISA)

The supernatant in routine effusion analysis is usually discarded, and some significant information is ignored, such as soluble markers, free DNA, and RNA. ELISA is an effective, simple method to detect soluble markers in the supernatant, which is valuable for diagnosis, treatment, and prognosis [94-97]. The basic process is to centrifuge the fluid, draw the supernatant, aliquot and freeze the supernatant at -80°C for later analysis, and then exploit commercially available ELISA kits for the test according to the manufacturer's steps [94, 95].

Singer G et al. detected a significant increase in the level of secretory HLA-G (sHLA-G) in malignant ascites by ELISA, indicating that sHLA-G can be applied to distinguish between benign and malignant effusions [96]. The combined detection of effusion and blood markers provides a new idea of diagnosis and treatment. Liu D et al. employed ELISA to test the levels of human epididymis protein 4 (HE4) in ascitic supernatants and corresponding CA125 in the serum of ovarian cancer patients with chemotherapy and non-chemotherapy; a positive correlation was shown between the 2 markers, and a high level of HE4 may predict chemotherapy resistance and ascites formation [97].

Flow cytometry

ICC is widely accepted in the diagnosis of effusion except for specimens with limited cells, and the flow cytometry serves as an important complementary technique. Although flow cytometry is mainly used for the diagnosis and treatment guidance of hematopoietic malignancies, it is also chosen for cell detection and DNA ploidy analysis of malignant effusions [98-101]. Effusion samples used for flow cytometry are fresh, unfixed, or stored in 10% DMSO/RPMI medium at -80°C, and the general steps are as follows [28, 99, 102]: after centrifugation, the supernatant is discarded; a filtration procedure can be added to remove large cells clumps or viscous aggregates; the pellet is washed and resuspended with RPMI or phosphate buffer saline to adjust the number of cells for analysis; and cells prepared for immunotyping should be placed on ice with a gentle operation to reduce debris or dead cells.

Examples of ordinary markers are Ber-EP4, CD45, CD14, N-cadherin, EMA, MUC4, progesterone (PR), and TTF-1 [99, 101-103]. An advantage of flow cytometry is the rapid multiparametric analysis of both surface markers and DNA aneuploidy, with no observer error [99]. Flow cytometry is a valuable method because it can provide immunotyping information to distinguish between benign and malignant cells and develop tailored treatment protocols; moreover, the fluorescence-activated cell sorting can highly purify tumor cells for subsequent molecular analysis [102]. In addition, the flow cytometry can quantitatively analyze the number of receptors per cell and whether the receptor protein is functional, by using fluorescently labeled ligands [28].

Molecular analysis

In an era of personalized medicine, targeted therapy has become a research hotspot. Individualized treatment plans based on the results of molecular tests help patients significantly improve progression-free survival and overall survival, providing them with substantial clinical benefits [104]. Cytological materials play an important role in molecular analysis because of their great accessibility, minimal invasion, safety, low cost, and easy patient acceptance [105, 106]. Both the supernatant and cellular components of the effusion can be tested for nucleic acids, proteins, and other molecules. The relevant information can be employed to diagnose, predict therapeutic responses, assess prognoses, and identify new therapeutic targets [3].

The FFPE-CB is the preferred preparation for molecular tests, but some laboratories adopt methanol fixation as an alternative because formalin fixation may cause the cross-linking of nucleic acids and proteins, and fragments and sequence artifacts, to interfere with detection [107, 108]. Other preparations such as smears exhibit a prior quality of DNA, and provide excellent resources for molecular analysis [109, 110].

Current molecular strategies include polymerase chain reaction (PCR)-based assays, sequencing, and fluorescence in situ hybridization (FISH) [72, 111-134] (**Table 3**). The choice of these methods is determined by the target gene/genes, mutation spectrum of the interested gene, sample size for screening, and available equipment [115]. The basic steps of molecular testing are as follows [83, 135, 136]:

Table 3. Comparison of different molecular analysis

Molecular tests	Basic principles	Strength	Shortcoming	Ref
PCR-based assays	Primers targeting specific genes for multiple copy analysis	Quick, simple, reproducible, sensitive, specific	Limited number of detected genes	[115, 121, 127, 131]
Sanger sequencing	Sequencing by termination	Detecting multiple types of mutations	Limited number of genes detected every single time Low sensitivity Requiring at least 20% of tumor cells	[114, 115, 118, 122]
Pyrosequencing	Sequencing by synthesis	Sensitive Requiring malignant cells as low as 5%	Limited number of genes detected every single time	[114, 122]
Next-generation sequencing (NGS)	Sequencing by synthesis	High-throughput analysis Single or multiple gene analysis Requiring a low number of tumor cells	Expensive Complicated results analysis	[111, 114, 120, 122, 125, 126, 128]
FISH	Probes targeting specific chromosomal abnormalities	Direct visualization of cytological materials	Limited number of probes each test containing Not available in every laboratory	[72, 112, 113, 117, 132, 137]

select and mark the appropriate area on the slides, place them in xylene overnight or use a deep-freeze method (put the slides in the freezer at -20°C for 1-2 minutes) to remove the coverslip, and then transfer the material to a small tube for cells lysis and DNA extraction. When there are limited tumor cells in serous examples, macrodissection, manual microdissection, or laser-capture microdissection is recommended for cell enrichment [106].

PCR-based assays

PCR is adopted to detect specific mutations. The synthetically designed primers are linked to the target sequence, many copies of the original target sequence are generated through a multicycle amplification process [115]. Quantitative real-time polymerase chain reaction (qRT-PCR) quantifies the number of target sequences in a sample by detecting the amount of fluorescent signal released in each cycle [127]. Rocco Cappellesso et al. exploited qRT-PCR to assess the expression levels of several microRNAs in cytological specimens, and the result showed that the combination of miR-21 and miR-126 achieved 86% sensitivity and 87% specificity in distinguishing malignant mesothelioma from reactive mesothelial cells [131]. Reverse transcription polymerase chain reaction (RT-PCR) reverses mRNA to complementary DNA to detect the expression of interest genes [121].

Sequencing

Sanger Sequencing is "sequencing by termination", which randomly inhibits the extension process; then, different lengths of newly formed DNA fragments can be detected by an automatic reader after electrophoresis separation [115, 122]. This method, which has long been regarded as the gold standard for direct DNA sequencing, can detect multiple types of mutations [118]. The disadvantage of Sanger Sequencing is low sensitivity, limited ability to recognize gene copy number changes, and least 20% of genetically altered tumor cells is required [114, 122]. Pyrosequencing, as an alternative to Sanger Sequencing, is "sequencing by synthesis", which recognizes the additions of specific bases by chemiluminescence detection of pyrophosphate released in the DNA PCR [114, 122]. This sensitive method can detect mutations of genetically altered malignant cells as low as 5% [114].

Next-generation sequencing (NGS)

NGS is "sequencing by synthesis", using the target gene as the template to synthesize complementary chain, which is visualized by 4 specific fluorescently labeled nucleic acids to obtain information of the interest gene [116, 120, 122]. NGS is the hottest, flexible sequencing method that allows simultaneous analysis of multiple gene targets with a minimum amount of DNA [125]. Many commercially available narrow-spectrum combinations can replace PCR to detect specific genetic variation, and broad-spectrum combinations can be applied to analyze whole-exome sequencing, accounting for approximately 2% of the entire genome [124, 126]. Because the analysis of the NGS results is complicated, more investment is required in informatics to develop a more practical algorithm to convert digital information into a simple quantitative score [128].

Current guidelines recommend routine detection of epidermal growth factor receptor *(EGFR)* and v-raf murine sarcoma viral oncogene homolog B *(BRAF)* mutations, as well as *ALK* and *ROS1* rearrangements, in patients with advanced or metastatic NSCLC [111]. *EGFR* detection involves screening and targeted tests: screening tests detect all *EGFR* mutations and potentially new mutations, and targeted tests detect the exactly known mutations that are clinically available [114]. Screening methods include Sanger sequencing, Pyrosequencing, NGS, and High Resolution Melt Analysis, and the targeted assays are the Agena MassARRAY Oncocarta panel, the Cobas *EGFR* Mutation Test (Roche Molecular Systems), the Therascreen EGFR Kit (Qiagen), and SNaPShot (by Life Technologies/Applied Biosystem) [114]. Molecular tests for *ALK*, *ROS1*, and *RET* rearrangements include FISH and RT-PCR [114]. Two experiments have shown 85% consistency between FISH and RT-PCR when detecting *ALK* rearrangements [133, 134].

FISH

When the cytological results are negative or ambiguous, FISH serves as a valuable supplementary tool, substantially improving the sensitivity without affecting the specificity [113, 137]. FISH is an accurate method that targets specific chromosome abnormalities and monitors the genetic status of cells to directly visualize cytological materials [72, 113]. Various

cytological materials can be employed for FISH, including conventional or liquid-based preparations, Papanicolaou- or Giemsa-stained, or immunocytochemical slides [132]. Among them, the alcohol-fixed or air-dried smears are more suitable for FISH analysis than FFPE-CBs because the cross-linking of nucleic acids and proteins caused by formalin fixation affects DNA quality [107]. The basic steps of FISH are as follows: after selecting and marking the appropriate hybridization area, remove the coverslip, denature by protease, incubate with fluorescently labeled specific DNA probe overnight, and then wash and observe at an appropriate wavelength after counterstaining the cell nuclei with DAPI [132].

EMA, desmin, and other immunocytochemical markers have limited ability to distinguish between reactive mesothelial cells and malignant mesothelioma in some cases, and molecular tests can identify several frequent genetic abnormalities in mesothelioma [112]. Among them, homozygous deletion of *9p21* is a relatively general alteration [119, 123, 129, 130], which can be detected by the commercially available UroVysion FISH kit. The 4 probes of the kit can hybridize to the centromere region on chromosomes 3, 7, and 17 and to the *p16 INK4A* gene locus at 9p21 [113]. The UroVysion FISH kit can be applied with cytology to obtain better diagnostic performance for effusion diagnosis, as R. DCB et al. showed [117]. They exploited this kit to 70 samples of pleural and peritoneal fluids; the final diagnostic sensitivity, specificity, and accuracy were 87.3%, 71.4%, and 85.7% respectively, and the results after combining with cytology were 88.0%, 83.3%, and 87.8%.

Moreover, FISH can be exploited to detect *ALK*, *ROS1*, *RET* gene rearrangement and *MET* amplification to screen lung cancer patients suitable for targeted chemotherapy, and it has been employed in urinary cytology for the diagnosis of urothelial tumors [132].

Conclusion

Serous effusions can be classified into benign and malignant. Cytological specimens are important materials because of their great accessibility, minimal invasion, and easy patient acceptance. Both the cellular components and supernatant of collected effusions are informative for precise and timely diagnosis, treatment, and prognosis.

Various methods are performed for the different diagnostic purposes of fluid samples. Morphological analysis is the basis. ICC assists in identifying morphologically ambiguous cases and provides possible organ origin and treatment-related information. EM is only applied as a supplement to ICC in the diagnostic dilemma of mesothelioma specimens. ELISA is employed to quickly detect soluble markers in the supernatant. Flow cytometry is mainly exploited in the diagnosis of hematopoietic abnormalities, allowing rapid multi-parameter analysis of cell surface markers and DNA aneuploidy, especially in samples of limited cellularity. In the era of precision medicine, molecular detection has gained increasing attention, which escorts the tailored targeted therapy. Among them, NGS is the hottest for high-throughput analysis with a minimum amount of DNA. Preclinical tumor models and molecular analysis are combined to provide useful information. New molecular detection and related technologies continue to emerge and make the diagnosis of effusion more accurate, efficient, convenient, and economical than ever before.

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