In this brief review, the role of various ancillary techniques to detect malignancy in effusion fluid are evaluated and discussed. The data were collected from a large number of research articles published in various medical journals. The role of these techniques to increase the diagnostic accuracy in serous effusions is emphasised.

The cytological diagnoses of serous effusions are usually made by routine cytomorphology with certainty, allowing treatment decisions. Various studies have shown a sensitivity of 57.3% and specificity of 89% by conventional cytology for the detection of malignant cells in effusion samples. Studies have shown that positive and negative predictive values for detection of malignancy by cytomorphology are 89.3% and 69.4% respectively. However, a grey zone always exists, where the cytopathologist encounters problems in determining the nature of the cells whether reactive, atypical, or beyond doubt malignant. Therefore various ancillary techniques should be used to increase the diagnostic accuracy of malignancy in serous effusions. The various ancillary techniques and markers are:

- Immunocytochemistry.
- Electron microscopy.
- Argyrophilic nucleolar organiser region.
- Flow cytometry.
- Image morphometry.
- Cell proliferation indices.
- Tumour markers.
- Marker of metastasis.
- Immunofluorescence.
- Telomerase activity.
- Polymerase chain reaction.
- Flow cytometry.
- Blood group antigens.
- HLA antigens.
- p53 oncogene product.

**IMMUNOCYTOCHEMISTRY**

The cytological differentiation of reactive mesothelial cells from adenocarcinoma metastatic to serosal membranes is often difficult. Although histochemical stains such as mucicarmine, periodic acid-Schiff with and without diastase digestion, and hyaluronidase alcin blue (or colloidal iron) stains are helpful, their usefulness is limited by their lack of specificity and sensitivity. Therefore immunocytochemical analysis is the most commonly used technique and often involves the use of a panel of antibodies. There is a great volume of literature emphasising the value of immunocytochemistry in diagnosing mesothelioma from metastatic adenocarcinoma. Differences of opinion exist regarding which markers should be included in the routine diagnostic panel for differentiation of them.

The various markers expressed by adenocarcinoma include carcinoembryonic antigen, B72.3, Ber-Ep4, M0C-31, low molecular weight cytokeratins, and BG-8 anti-Lewisy. A diagnosis of metastatic adenocarcinoma is favoured if the neoplastic cells demonstrated immunoreactivity for the aforementioned markers; however, these markers are not distributed uniformly among different types of adenocarcinomas. Positive staining with at least two adenocarcinoma markers would favour the diagnosis. Therefore, a panel of antibodies is often used to confirm or to exclude the possibility of an adenocarcinoma.

Specific mesothelial cell markers include ME1 monoclonal antibody, OV-CAR3, HBME-1, N-cadherin, and calretinin. In addition, mesotheliomas show positivity for both low and high molecular weight cytokeratins. The epithelial mesothelioma expresses thrombomodulin ranging from 49.1% to 100% positivity in different series. Similarly, K1 monoclonal antibody generated by immunising mice with the OV-CAR3 ovarian cell line, reacts with both epithelial and biphasic mesotheliomas. HBME-1 and mesothelial cell microvillus surface antigen are expressed by more than half of the mesothelial cells, however, more than half of adenocarcinomas also show similar immunoreactivity. N-cadherin is immunoreactive with 77% of reactive mesothelial cells, 35% malignant mesotheliomas, and 48% of adenocarcinomas. Several investigators have demonstrated that calretinin is a sensitive and specific marker for both reactive and malignant mesothelial cells. But, unfortunately, scattered positive staining for calretinin is also reported in 5% to 10% of adenocarcinomas. Therefore, immunocytochemical profile of positive staining

**Abbreviations:** AgNOR, argyrophilic nucleolar organiser regions; EGP-2, epithelial glycoprotein-2; MMP, matrix metalloproteinases; NOR, nucleolar organiser regions; PCR, polymerase chain reaction; RT, reverse transcriptase; VEGF, vascular endothelial growth factor
with any adenocarcinoma markers and negative staining with calretinin are specific and sensitive for recognising adenocarcinoma in fluid cytology. Table 1 summarises the positivity of different immunostaining for confirmation of mesotheliomas and adenocarcinomas.

**ELECTRON MICROSCOPY**

Ultrastructural examination of the mesothelioma cells show slender and bushy microvilli devoid of glycosylated bodies, evenly distributed around the entire cell surface, whereas in adenocarcinoma these are short and stubby with glycosylated bodies concentrated at poles. In addition, mesothelial cell shows tonofibrils surrounding the nucleus, abundant glycogen, and apical tight junctions with well developed desmosomes. However, electron microscopy is costly and adequate tissue for ultrastructural evaluation may not be available and the turnover time of electron microscope is quite lengthy. Furthermore, there may be overlapping features with adenocarcinoma and sometimes the interpretation may be difficult.

**ARGYROPHILIC NUCLEOLAR ORGANISER REGION**

The argyrophilic nucleolar organiser region (AgNOR) technique detects specialised nucleolar protein using a silver staining method. The number and size of NORs reflects cellular proliferation, activity, and transformation and may help to differentiate benign from malignant cells. AgNOR associated proteins have widespread application in diagnostic pathology. The reliability of this method in the evaluation of malignancy has been frequently demonstrated even by a simple visual assessment. Thus, counting of AgNOR dots appears to be a very useful and simple way of obtaining data on the proliferative index of malignant as well as benign lesions. Various workers have applied this technique in serous effusions to detect malignancy. Sujathan et al have shown higher AgNOR counts in malignant cells, mean (SD) 4.72 (0.76), compared with reactive mesothelial cells, mean (SD) 1.92 (0.23), in serous effusion samples. In addition, the NORs are irregular in shape in cases of malignant cells, while these are larger and appear as single dots in reactive mesothelial cells. Subsequently, Rocher et al have shown significantly higher NOR counts in malignant cells, mean (SD) 13.78 (3.88), compared with reactive mesothelial cells, mean (SD) 4.88 (1.5). Later on Pomjanski et al compared the results of conventional cytology, DNA image cytometry, immunocytochemistry, and AgNOR counts for the diagnosis of malignant cells in serous effusion. With the help of the AgNOR count, they detected 73% of malignant mesotheliomas on effusions.

**FLOW CYTOMETRY**

Flow cytometry has been a rapidly emerging technology over the last few decades. This expensive and sophisticated technology is being used increasingly from research laboratories to clinical laboratories. DNA flow cytometry requires single cells or nuclei in fluid suspension. The dissociated cells are stained with a DNA specific dye. These dyes bind with DNA stoichiometrically. The stained cells pass single in front of the laser beam. The cell absorbs the light and emits fluorescence, which is measured by flow cytometry with the help of a photomultiplier tube. The emitted fluorescence is proportional to the DNA content of the cells and is represented as a channel number, which is an arbitrary value depending on the machine’s initial set up. The data are displayed as a DNA histogram. A DNA histogram thus obtained from the peripheral blood lymphocytes should show a single peak as all the cells contain 46 chromosomes (2n). Another small peak in double the channel number is also found, which represents the G2-M phase of cells. These cells contain 2×46 (4n) chromosomes. The cells in between the two peaks usually represent the S-phase of the cell cycle. Any peak, other than these two peaks, should be considered as an aneuploid peak. DNA flow cytometry is commonly used for the diagnosis and prognosis of tumours. DNA aneuploidy and high S-phase (proliferative fraction) percentage are commonly noted in malignant tumours and may be used for diagnosis of malignancies.

The technique of DNA flow cytometry has been used in effusion fluid by various workers to detect malignancy and showed wide variation in sensitivity and specificity. Saha et al have shown 99% sensitivity and 99% specificity of flow cytometry in effusion samples, whereas Evans et al have shown 100% sensitivity and 86% specificity. The wide variation of sensitivity (55%–100%) and specificity (86%–100%) of DNA flow cytometry may be explained by the number of cases examined, types of cases included, criteria of aneuploidy, and various ways of processing of specimen for flow cytometry. Considerable admixtures of benign mesothelial cells with scanty malignant cells may create a problem in getting a prominent aneuploid peak. Dual colour multiparametric flow cytometry may be helpful to increase the sensitivity of flow cytometry.

Flow cytometric determination of the percentage of natural killer lymphocytes can be useful to diagnose the metastatic effusion. Studies by Green and Griffin and Laurini et al have shown this as a quick procedure that appears to have a high sensitivity (100%) and specificity (96.8%) for the diagnosis of malignant effusions due to metastatic adenocarcinoma.

**IMAGE MORPHOMETRY**

Computerised image analysis can rapidly digitise the image of the cells and thereby can estimate various morphometric parameters of the cell. Cytological samples suitable for image can be obtained from smears, touch preparations, or cytocentrifuge. The various cellular parameters that can be measured by image analysis include nuclear as well as cytoplasmic diameter, circumference, area, nuclear shape, ratio of nucleus to cytoplasmic area, chromatic texture, percentage positivity on immunocytochemistry, and optical density (sum optical density for DNA estimation by Feulgen stain).

### Table 1 Immunocytochemistry of mesothelioma and adenocarcinoma

<table>
<thead>
<tr>
<th>Immunological markers</th>
<th>Mesothelioma</th>
<th>Adenocarcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinoembryonic antigen</td>
<td>Consistently negative</td>
<td>Consistently positive</td>
</tr>
<tr>
<td>Epithelial membrane antigen</td>
<td>Occasionally positive with thick membranous pattern</td>
<td>Cytoplasmic and membranous positive</td>
</tr>
<tr>
<td>Low and high molecular weight keratin</td>
<td>Both positive</td>
<td>Only low molecular weight keratin positive</td>
</tr>
<tr>
<td>B 72.3, Ber-EP4, Leu M1</td>
<td>Consistently negative</td>
<td>Consistently positive</td>
</tr>
<tr>
<td>Calretinin, HBME-1, N-cadherin</td>
<td>Positive</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Have I spelt out EMA correctly in table 1?
**Compared with DNA flow cytometry, image cytometry is advantageous when the number of cells obtained for examination is limited.**67 Cells can be selected in an automated fashion based on cellular characteristics. In a recent study, automatic cell differentiation was performed on cells stained by haematoxylin and eosin.68 In this set up, cytoplasmic as well as nuclear features were used in the selection algorithm. In another study, Thunnissen et al examined the diagnostic value of DNA image cytometry for automatic cell selection in serosal fluid cytology.69

**Proliferation markers**
The diagnostic accuracy of malignant effusions can be improved by employing various cell proliferation markers.70 MIB1 monoclonal antibody (Ki67) is present in cycling cells, but not in resting cells. Estimation of the percentage of cells reacting with Ki67 immunocytochemical staining can be performed counting 1000 cells on a consecutive high magnification field.51–55

Saleh et al have shown a statistically significant correlation between the Ki67 index and cytomorphology of benign (9%), suspicious (19%), and malignant (28%) cases.60 However, cytomorphology should always remain the basis to differentiate benign from malignant serous effusions and Ki67 stain is a valuable adjunct in difficult cases, acting as a complementary tool to routine cytology.

**Tumour markers**
Malignancies of any organ can metastasise to the serosal cavities, but the most common cancers concerned are lung and breast carcinomas, lymphoma, and less frequently, digestive and ovarian malignancies.54–56

Cytology alone has its limitations in certain cases in diagnosing malignancy. Many researchers have investigated the assay of tumour markers in effusion fluid in order to improve the diagnostic yield.54–56 Carcinoembryonic antigen has been extensively studied and has shown a diagnostic sensitivity of about 50–60%.73 Nevertheless, for a particular carcinoma, the use of a single tumour marker may be insufficient because it may not be uniformly expressed to detect all types of malignancies. Thus many studies emphasise the use of carcinoembryonic antigen along with various tumour markers such as carbohydrate antigens 15–3, 19–9, and 72–4. CYFRA 21–1, a serum assay for soluble fragments of cytokeratin 19, has been recently proposed for the diagnosis and the follow up of non-small cell lung carcinoma, squamous cell carcinomas of the head and neck, uterine cervix, and bladder cancer.54,56–58 Salama et al have shown high values of this marker in the pleural fluid of patients with mesothelioma.59

**Marker of metastasis (CD44 and CD44v isoforms)**
CD44 is a widely distributed integral membrane protein that exists in a variety of forms with different molecular sizes ranging from 85 kD to 160 kD.80 It acts as a receptor for hyaluronic acid and important in lymphocyte homing.81 CD44 isoforms are observed in epithelial malignancy with high metastatic potentials. The detection of malignant cells in serous effusions obtained from patients diagnosed with cancer indicates the presence of metastatic disease and furthermore is associated with poor biological outcome. Berner et al assessed the role of CD44s and CD44v isoforms (CD44v3, v5, v6, v7, and v3–10) to distinguish mesothelial cells and malignant epithelial cells in effusions using immunocytochemical technique.82 The percentage immunoreactivity for CD44s was more in benign mesothelial cells (94%) compared with malignant/atypical epithelial cells (23%) and in contrast CD44v3–10 positivity was more in malignant/atypical cells (55%) than benign cells (6%). Therefore, as a marker of metastatic disease CD44s and CD44v isoforms, particularly CD44v3–10, can be used in a difficult situation.

**IMMUNOFLUORESCENCE**
The immunofluorescence double staining technique can be applied in malignant effusions to combine DNA measurement with those of immunocytochemical and ligand immunocytochemical reactivity. Kayser et al investigated potential disease and prognosis associated nuclear and cellular features from cell properties in a prospective study on malignant pleural effusion.73 They measured the integrated nuclear fluorescence, the expression of binding capacities of carrier immobilised oestradiol, progesterone, testosterone, sarolectin, and the presence of calcyclin in 50 cases of proven malignant pleural effusion (10 mesotheliomas and 40 metastatic tumours pleural effusion). A significant correlation was obtained between the S-phase related tumour cell fraction and the expression of progesterone receptor.

**Telomerase activity**
Telomeres are specialised structures at the ends of the chromosomes in eukaryotic cells. They are shortened with each cell division, finally resulting in cellular senescence. The enzyme, telomerase, a ribonucleoprotein, compensates for telomeric loss. Telomerase is believed to play an important part in the evolution of various malignancies. A polymerase chain reaction (PCR) based method is used to determine telomerase activity.74 Telomerase activity has been demonstrated in various malignancies, which include gastric, breast, prostatic, cervical, and so on.75–79 In pleural effusions, telomerase activity was detected in 52%–91% of specimens diagnosed as malignant in routine cytology.75–83 However, Dejmek et al have shown telomerase activity in 67% cases of malignant effusion.74 Telomerase is found to be one of the promising markers of malignancy, however, the reactive atypical cells also show nuclear fluorescence. In this situation, the cells strongly suspicious for malignancy on cytology should be taken into account.

**Polymerase chain reaction**
The PCR enables exponential amplification of DNA or RNA sequences enhancing diagnostic sensitivity and specificity using specific primer.84 Sakaguchi et al developed a sensitive and specific method for the detection of epithelial malignancy with a two stage molecular based assay that combined enrichment for cancer cells by immunomagnetic bead selection and reverse transcriptase (RT)-PCR detection of epithelial glycoprotein-2 (EGP-2) RNA.85 In their study, 110 cases of pleural and peritoneal effusions (30 from patients with known carcinoma and 80 from those without known carcinoma) were taken and the results were compared with cytomorphological features. Out of 18 cytologically positive or suspicious effusions, 17 (94%) were positive for EGP-2 RNA. The one negative sample was from a patient who had recently received combination chemotherapy. Of the 92 cytologically negative samples, 11 (12%) were positive for EGP-2. Therefore, this method appears to be highly specific and sensitive in detecting malignant cells and may be useful as an adjunct to routine cytomorphological examination. Davidson et al have analysed mRNA expression of matrix metalloproteinases (MMP) membrane type 1, 2, and 3 in serous effusions of patients with ovarian malignancies using the....
RT-PCR reaction. In addition they also evaluated membrane type 1 MMP expression in effusions in primary and metastatic lesions using mRNA in situ hybridisation. Membrane type 1 MMP was localised to tumour cells in 32 of 85 primary and metastatic solid lesions, and stromal cells expressed membrane type 1 MMP in three cases.

**FLUORESCENCE IN SITU HYBRIDISATION**

In recent years interphase cytogenetics by fluorescence in situ hybridisation (FISH) has been used in clinical pathology to delineate chromosomal aberrations in neoplasia. FISH is a powerful tool for genetic evaluation and permits microscopic identification, localisation of aberrations in interphase as well as metaphase of the cell cycle. Fiegler et al in their study have shown the role of FISH in detecting the malignant cells in effusion fluid from patients with carcinoma. Their study included 201 effusions from patients with advanced cancer, along with nine with a primary breast tumour. They have used various centromeric probes to determine the malignancy associated changes.

Subsequently, another study has shown a hyperdiploid cell population in pleural effusion fluid using dual coloured FISH. Therefore, the FISH technique can be used in effusions along with cytology to detect the aneuploid cells indicating malignancies.

**GROWTH FACTORS**

Vascular endothelial growth factor (VEGF) is an important mediator of angiogenesis. Zebrowski et al have shown significantly higher VEGF levels in malignant pleural effusion by using an immunoassay. Yanagawa et al have shown a significantly higher amount of VEGF by enzyme immunoassay in cytologically proven malignant pleural effusions associated with primary lung cancer than in those with benign exudative pleural effusions. However, none of these studies has highlighted the role of VEGF in differentiating metastatic effusion from mesothelioma.

Lysophosphatidic acid and sphingosine-1-phosphate are bioactive phospholipids with mitogenic and growth factor-like activity. They act through a specific cell surface receptor, which is located in many normal as well as transformed cells. Lysophosphatidic acid has recently been implicated as a growth factor in ovarian cancer patients in effusions. Westermann et al postulated a role of lysophosphatidic acid-like lipids in the peritoneal spread of ovarian cancer and possibly that of other predominantly intraperitoneal malignancies.

**BLOOD GROUP ANTIGENS**

Sialosyl-Tn is an aberrantly glycosylated precursor of the MN blood group antigen frequently expressed in carcinomas and dysplastic epithelium. This can be assessed by solid phase immunoradiometric assay and immunocytochemistry. Zimmerman et al showed that sialosyl-Tn may have diagnostic value in discriminating carcinoma cells from reactive mesothelial cell in serous effusions. They studied sialosyl-Tn immunocytochemistry on cell block material from 72 serous effusion samples. The immunoreactivity was strong in carcinoma (77%) cases compared with moderate to weak staining in benign (4%–18%) cases. The positivity and specificity values of sialosyl-Tn immunostaining were 100% and 78% whereas the positivity and negative predictive values were 100% and 76% respectively.

**ROLE OF HLA ANTIGENS**

Magyarosy et al showed significant paucity of HLA-1 antigens on metastatic carcinoma cells in effusion fluid, whereas the reactive mesothelial cells showed uniformly strong positivity for both HLA-1 and β₂-microglobulin. Various metastatic carcinoma cells can be identified in effusion fluids by appropriate immunocytochemical stains.

**ONCOGENE PRODUCT**

p53 is a nuclear phosphoprotein that appears to play an important part in regulating cell death by apoptosis and thereby net cell increment. It is well accepted that wild type p53 protein acts as a “tumour suppressor” in normal cells. Mutation of the p53 tumour suppressor gene occurs frequently in mesotheliomas, lung, and colonic carcinomas. Mutated forms of p53 have a longer half life than wild type p53 and therefore accumulated in the nucleus. This mutated p53 is readily detectable by immunohistochemical methods. Various studies have shown that p53 immunostaining on effusion can detect 50%–55% of malignancies in effusion. Benign cells are negative for p53 immunostaining. Various authors have concluded that p53 immunostaining is highly specific and moderately sensitive marker of malignancy in effusion fluid.

<table>
<thead>
<tr>
<th>Ancillary tests</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunocytochemistry</td>
<td>Commonly used Panel of antibody is needed High sensitivity and specificity Cost effective</td>
</tr>
<tr>
<td>Electron microscopy</td>
<td>Costly Long turnover time Helpful to distinguish mesothelioma and adenocarcinoma</td>
</tr>
<tr>
<td>AgNOR</td>
<td>Simple and easy to do Cheap Have potential value</td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>Costly Variable sensitivity Diploid pattern does not rule out malignancy and rarely benign cells show aneuploidy Dual colour flow cytometry more helpful</td>
</tr>
<tr>
<td>Image cytometry</td>
<td>Visual light microscopic selection of cell possible Slow and tedious Laser scanning image cytometry is rapid</td>
</tr>
<tr>
<td>Proliferative markers</td>
<td>High Ki67 index is an excellent marker to recognise rapidly proliferating cell population indicating malignancy</td>
</tr>
<tr>
<td>Tumour markers</td>
<td>Helpful in selective tumours to diagnose</td>
</tr>
<tr>
<td>Markers of metastasis</td>
<td>CD44v 3–10 positivity may be helpful in difficult situation</td>
</tr>
<tr>
<td>Immunofluorescence</td>
<td>Experimental stage Integrated nuclear fluorescence of oestriadiol and progesterone may be helpful</td>
</tr>
<tr>
<td>Telomerase activity</td>
<td>Demonstration of telomerase activity may indicate malignancy</td>
</tr>
<tr>
<td>PCR</td>
<td>Detection of EGF-2RNA by RT-PCR is specific and sensitive</td>
</tr>
<tr>
<td>FISH</td>
<td>Minor chromosomal aberration can be detected No chromosomal culture needed</td>
</tr>
<tr>
<td>Growth factor</td>
<td>VEGF, an indicator of angiogenesis, is significantly higher in malignant effusion</td>
</tr>
<tr>
<td>Blood group antigen</td>
<td>Sialosyl-Tn expressed in carcinomas High sensitivity and moderate specificity</td>
</tr>
<tr>
<td>p53</td>
<td>Mutant type p53 is demonstrable in malignant cells High specificity but low sensitivity</td>
</tr>
</tbody>
</table>
Table 2 summarises the salient features of different ancillary techniques to identify malignancy in effusion fluid. The judicious application of these techniques is needed to increase the diagnostic accuracy and to make a decision. Many of these techniques are at an experimental level and quite promising. Furthermore the cost effectiveness of these techniques should also be taken into consideration for their future application in a clinical laboratory.

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