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# Immunocytochemistry of effusions: Processing and commonly used immunomarkers

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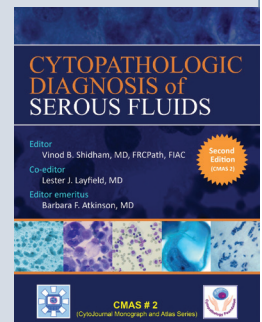
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1. Introduction
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5. Immunocytochemistry of effusion fluids: introduction to SCIP approach
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11. Serous cavity metastasis: Evaluation of unknown primary
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13. Flow cytometry, molecular analysis, and other special techniques
14. Appendix I: Collection and processing of effusion fluids for cytopathologic evaluation
15. Appendix II: Immunocytochemistry of effusions: processing and commonly used immunomarkers

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## ABSTRACT

Definitive cytopathological interpretation of some of the effusion fluids may not be possible based on cytomorphological evaluation alone. As discussed in other reviews, this is due to various reasons specifically applicable to effusion fluids including remarkably wide morphologic spectrum of reactive mesothelial cells overlapping with some well to moderately differentiated metastatic carcinoma. The challenge is subject to various factors including level of interpreter training or experience, institutional demographics (such as type of prevalent diseases, predominant sex and age group), technical advances in ancillary support, and expertise in cytopreparatory processing. In such cases immunohistochemistry performed on cell-block sections is simple objective adjunct with or without other ancillary techniques. Ongoing increase in number of immunomarkers along with rabbit monoclonal antibodies with relatively higher affinity is further refining this field. SCIP (subtractive coordinate immunoreactivity pattern) approach, discussed as separate dedicated review article, facilitates refined interpretation of immunoreactivity pattern in coordinate manner on various serial sections of cell-blocks. However, many variables such as delay after specimen collection, specimen processing related factors including fixation and storage; ambient conditions under which paraffin blocks are archived (for retrospective testing); antigen retrieval method; duration of antigen retrieval step; antibody clone and dilution; and antibody application time are common with application of immunohistochemistry in other areas. This review is dedicated to highlight technical aspects including processing of effusion specimens for optimum immunocytochemical evaluation along with commonly used immunomarkers in effusion cytopathology. This review focuses on the technical and general information about various immunomarkers.

**Keywords:** Immunohistochemistry, IHC, immunocytochemistry, immunostaining, immunomarker, immunoreactivity, immunocharacterization, effusion fluids, SCIP approach, monoclonal antibodies

Immunophenotyping of cells in effusions improves their objective analysis of metastatic cancer cells. For consistent and reproducible results, proper processing of effusions is extremely crucial. This appendix highlights some of the technical aspects related to the processing of effusions for optimum immunocytochemical evaluation by the 'subtractive coordinate immunoreactivity pattern' (SCIP) approach, along with a brief description of some immunomarkers related to the effusion immunocytochemistry.<sup>[1,2]</sup>

## SPECIMEN PROCESSING

• *As in surgical pathology, formalin-fixed paraffin-embedded cell-block section, although not ideal, are preferred for immunocytochemical evaluation. The use of other fixatives or preservatives may alter antigenic properties and cause misleading non-reproducible results.*

Although cell-block sections are preferred and considered optimum, depending on the practical situation, immunocytochemistry may be performed on different types of cytology smears, including direct smears, Cytospin smears,<sup>[3-6]</sup> and liquid-based cytology preparations (SurePath and ThinPrep), etc.<sup>[4-6]</sup> However, with this approach, various limiting factors, such as possible interference with immunoreactivity, increased background staining, and lack of opportunity to evaluate coordinate immunoreactivity in the same cells (NOT just similar cells), should be considered in the final interpretation of results. • *The results obtained are generally compared to those reported in the literature, which are predominantly based on immunocytochemistry with formalin-fixed paraffin-embedded tissue sections. This is the most important caveat to be considered when using cytology smears for immunocytochemistry of effusions, since the results obtained may not be reproducibly comparable to those that are published.*

## CYTOLOGY SMEARS

When the sample is scanty, immunocytochemistry may have to be performed on smears. Direct smears, Cytospin smears, or liquid-based cytology preparations (SurePath and ThinPrep) may be standardized for immunostaining, as indicated below.

### Direct smears

Direct smears are prepared by spreading the sediment of the specimen obtained by centrifugation on a slide. They may be processed and stained in a manner similar to a Cytospin smear, as described below.

### Cytospin smears

The Cyto centrifuge (Shandon, Pittsburgh, PA) is used at 500 rpm for 5 minutes to prepare Cytospin smears. Charged or 'silanated' microscope slides (Fisher Superfrost Plus; Fisher Scientific, Pittsburgh, PA) are recommended. Albuminized slides may cause non-specific background staining and are not preferred. To increase the cell adhesion and prevent the loss of cellular material during immunostaining, the smears are air-dried for 30 minutes at room temperature.

At least 100 dispersed neoplastic cells without significant overlap should be present on a slide. The cell density may be adjusted with RPMI-1640 (Gibco BRL, Grand Island, NY) or other isotonic medium such as Isotonic Medium S™ (AV BioInnovation, MI, USA).<sup>[7]</sup> For hemorrhagic effusions, the red cells may be separated out by the Ficoll technique (Accu-Prep Lymphocytes, Westbury, NY) or lysed with lysing agent used for flow cytometric immunocharacterization with ammonium chloride based lysing agents such as BloodLyz™ (AV BioInnovation, MI, USA).<sup>[8]</sup>

Fixation of the Cytospin smears is performed immediately prior to immunostaining. For any new antibody as an

immunomarker for immunocytochemistry, optimal immunoreactivity should be examined with reference to a variety of fixatives (i.e. acetone, ethanol, formalin, or paraformaldehyde), smear preparation methods (wet-fixed vs air-dried fixed vs post-fixed after saline rehydration of air-dried smears), fixation times, and any other variables.<sup>[4,6,9-19]</sup> The manufacturer's antibody specification sheet may provide some of this information. Some variables may have anticipated interference; e.g. longer fixation time may decrease sensitivity of detection of some antigens. For lymphoma immunomarkers, 10 minutes fixation in acetone at room temperature is optimum, whereas immunomarkers for epithelial cells/carcinoma cells need 5 minutes in alcohol (either 95% ethanol or, preferably, a 1:1 mixture of absolute methanol and absolute ethanol) at room temperature.<sup>[4]</sup> The fixed smears are thoroughly air-dried before proceeding with the immunostaining. For nuclear antigens, most protocols require 10–15 minutes fixation in either 3.7% buffered formaldehyde or 4% paraformaldehyde. Usually this is followed by a brief membrane permeabilization step using a dilute solution (0.25%) of Brij or Triton (Sigma Chemical Co., St Louis, MO). Alternatively, this can be achieved by fixation in cold methanol followed by cold acetone. At this stage the smears should not be allowed to air-dry before proceeding with the immunostaining.<sup>[4]</sup>

• **Air-dried saline rehydrated smears, fixed in alcoholic formalin, showed the best results with most immunomarkers except vimentin.**<sup>[16-18]</sup> The immunoreactivity of each immunomarker is affected by the method of smear processing.<sup>[14-16]</sup> Air-dried saline rehydrated smears fixed in 95% ethanol with 5% acetic acid, though a desired routine choice for cytomorphologic Papanicolaou (PAP) staining, are not suitable for immunostaining.<sup>[16-19]</sup>

Air-dried unfixed smears may be stored under cool dry conditions until they are processed for immunostaining. They may be stored in a plastic microscope slide storage box, which may be sealed in an airtight plastic bag with a desiccant such as Drierite (W.A. Hammond Drierite Co., Xenia, OH). These sealed smears may be stored in a refrigerator for at least 2 weeks without deteriorating.<sup>[15]</sup> The smears may be removed only after they are brought to room temperature; otherwise, condensation will build up on the slide and damage the unfixed cells.

#### Liquid-based cytology (LBC) smears (SurePath or ThinPrep)

Immunocytochemical evaluation may be performed on LBC smears by standardizing a protocol for specific immunomarkers. A few studies have used LBC for immunocytochemistry of other types of specimens.<sup>[20-22]</sup> If available, general guidelines suggested by the manufacturer for processing and developing protocols for immunostaining of LBC may be followed.

## CELL-BLOCKS

• **Most published studies comparing immunophenotypes are based on results obtained with formalin-fixed paraffin-embedded tissue sections. Because of this, formalin-fixed paraffin-embedded cell-blocks are recommended for immunocytochemical evaluation of effusions.**<sup>[6,23]</sup>

Substituting formalin or altering any of the processing steps may adversely affect the immunoreactivity pattern and lead to non-representative results.<sup>[24]</sup> For an individual case, the immunoreactivity may be enhanced by a particular approach with excellent crisp immunostaining, but this may not be comparable with the results in the literature and may be responsible for false-positive interpretation. At the other end of the gamut is loss of immunoreactivity by the modified protocol, which leads to false-negative results.

The pattern of malignant cells in effusions varies from the small groups of cells to the scattered isolated cells. Singly scattered abnormal cells in 3–4  $\mu\text{m}$  serial sections may be difficult to locate in subsequent sections. • **To overcome this, 3–4  $\mu\text{m}$  thick serial sections should be oriented and mounted in an identical manner on glass slides.**<sup>[1,2]</sup> The slides should be labeled serially, so that each section can be related to another by referring to the serial numbers on the slides. This simple SCIP approach is especially rewarding in unexpected situations especially when immunostaining pattern is not straightforward and needs careful scrutiny.<sup>[1,2]</sup>

Sections of cell-blocks (3–4  $\mu\text{m}$ ) are mounted on charge-coated slides and immunostained with a routine protocol following standard precautions and guidelines for quality control.<sup>[15]</sup>

## CELL-BLOCK VERSUS CYTOLOGY SMEARS [Direct Smears/Cytospins/Liquid based cytology preparations (SurePath and ThinPrep)] FOR IMMUNOCYTOCHEMISTRY

The most significant limitation with cytology smears is the inability to evaluate coordinate immunoreactivity, since the same cells cannot be present on more than one smear. On the other hand, serial sections of cell-blocks allow such evaluation of multiple immunomarkers in serial sections of the same cells.

Although cytologic preparations have been used successfully for immunostaining, they are not recommended for the immunocytochemical evaluation of effusions.<sup>[3]</sup> Complicating factors associated with effusion fluid immunocytochemistry include non-specific and unexpected immunoreactivity due to the protein-rich fluid in which the cells are floating, and large three-dimensional cell groups which may entrap immunostains, leading to false-positive results. Crushed, degenerated, or necrotic cells may show non-specific immunostaining.

## IMMUNOMARKERS

Over the past few years, the number and spectrum of antibodies available for immunocytochemistry have increased dramatically.<sup>[15,25]</sup> In addition, other technical advances have also enhanced the role of immunocytochemistry. The technique of heat-induced antigen epitope retrieval has remarkably improved immunostaining for a wide range of antibodies.<sup>[26–28]</sup> Automation and ready-to-use kits have further improved the reliability and the reproducibility of immunostaining. Quality assurance programs, participation in external proficiency testing, laboratory inspections (reinforcing appropriate documentation, equipment maintenance, procedure updating) and the requirement of qualified testing personnel have further improved the technical aspects of immunostaining.<sup>[29–31]</sup> In addition, recent availability of rabbit monoclonal antibodies has furthered the refinements in this technology.<sup>[32]</sup>

### GROUPING OF IMMUNOMARKERS FOR EFFUSION IMMUNOCYTOCHEMISTRY

The different immunomarkers used for effusions may be grouped by their predominant pattern of immunoreactivity:

#### Predominantly adenocarcinoma immunomarkers

BerEP4  
Claudin-4  
EMA--cytoplasmic  
B72.3  
mCEA  
CA19.9  
CD15 (not effective for effusions)

#### Miscellaneous adenocarcinoma immunomarkers

HMFG-2  
MOC-31  
Other organ-specific immunomarkers such as TTF-1, PSA, CDX2, GATA3, PAX8, PAX2, NKX3.1, etc.

#### Predominantly mesothelial immunomarkers

Calretinin  
Vimentin  
WT-1  
Cytokeratin 5/6  
Cytokeratin 7  
EMA (membranous with microvilli)  
HBME-1 (membranous with microvilli)  
D2-40 (podoplanin): new—promising role  
Mesothelin: new role evolving

#### Miscellaneous mesothelium-associated immunomarkers

CD44  
Thrombomodulin  
Antimesothelial cell antibody  
N-cadherin  
OV632

#### Loss of immunomarkers in mesothelial cells as diagnostic of mesothelioma

BAP1 (nuclear)

MTAP (cytoplasmic)

#### Miscellaneous with conflicting reports

E-cadherin  
HBME-1

### RECENT UPDATE:

#### Dual-Color Immunostaining to Improve Scip Approach<sup>[23,33,34]</sup>

Recently we evaluated dual-color immunostaining of effusion fluid cell-block sections.<sup>[33,34]</sup> Based on the categorization of different effusion immunomarkers mentioned above, various pairs of two immunomarkers may be applied. A few combinations mentioned below were evaluated (Table 1):

- A. BerEP4 (brown) followed by *Vimentin* (red) [Figure 1]
- B. *Vimentin* (brown) followed by cytokeratin 7 (red)
- C. *Calretinin* (brown) followed by BerEP4 (red)
- D. *Calretinin* (brown) followed by cytokeratin 20 (red)

*Combination A* (for immunostaining protocol, please see below) showed excellent results for detecting and identifying second foreign population of metastatic adenocarcinoma cells in most cases as brown immunostained cells on the red immunostained background (reactive mesothelial cells and inflammatory cells) (Figure 1).<sup>[33]</sup> Similar to BerEP4 brown immunoreactivity, the metastatic adenocarcinoma cells may also be standardized by replacing Claudin 4 as brown immunostaining step with vimentin red immunostaining.

*Combination B* correlated with the immunoreactivity pattern observed with single-color immunostaining. The original immunoreactivity pattern of the second immunomarker was compromised in *combinations C and D*. BerEP4 showed decreased immunostaining intensity with a negative result in some cases. Immunoreactivity for cytokeratin 20 was completely lost in *combination D*. This interference is under investigation, but initial findings suggest that the concentration of the second antibody (related to alkaline phosphatase system with red chromogen) should be higher than that used for the peroxidase system with 3,3'-Diaminobenzidine (DAB) as the chromogen.

On a scale of 1 (easy) to 5 (difficult), the average difficulty of interpretation was 2.95 (range 1–5) utilizing the single-color method.<sup>[34]</sup> However, interpretation with the dual-color method was very easy with an average difficulty score of only 1. This difference was statistically significant (two tailed p value <0.0001, paired t test). Higher scores of difficulty were observed in cases with scant tumor cells or predominantly single tumor cells, when the SCIP approach<sup>[2]</sup> with conventional one-color immunocytochemistry was applied. The interpretation of some of the specimens in this category was impossible, if the sections were not oriented identically on all slides and the serial relationship was not properly identified on individual slides.

The studies<sup>[1,2]</sup> concluded that dual staining facilitated easy identification of the foreign populations of malignant cells in effusion fluids. However, slightly decreased or complete loss of immunoreactivity for BerEP4 and complete loss for Cytokeratin (CK) 20 suggested a need for standardization of any combination to be applied with any particular protocol. The protocol used in this study is detailed below.

**Dual-color immunostaining protocol [example BerEP4 (brown)with vimentin (Red)]:**

Note:

- Staining performed on automatic Ventana BenchMark ULTRA IHC/ISH platform.
  - Unless indicated all reagents are from Ventana-Roche.
  - Slide is rinsed with Reaction Buffer ( pH 7.6).
  - Coverslip (Ultra LCS) solution is oil base solution, to prevent evaporation during high temperature incubation.
1. Deparaffinize the section with EZPrep solution (1:10) at 72°C.
  2. Apply Cell Conditioner # 1 and incubate for 36 minutes at 95°C.
  3. Apply one drop of UV INHIBITOR (part of ultraView Universal DAB Detection Kit), incubate for 4 minutes.
  4. Apply one drop of *First Primary Antibody* (Prep kit #11 BerEp4 , Dako), incubate for 32 minutes at 36°C.

5. Apply one drop of UV Horseradish Peroxidase (HRP) Universal Multimer (part of DAB Kit), incubate for 8 minutes.
6. Apply one drop of UV DAB (part of DAB Kit) and one drop of UV DAB H2O2 (part of DAB Kit), incubate for 8 minutes.
7. Apply one drop UV COPPER (part of DAB Kit), incubate for 4 minutes.
8. Apply one drop of *Second Primary Antibody* (Vimentin), incubate for 16 minutes at 36°C.
9. Apply one drop of UV RED UNIV MULT (part of ultraView Universal Alkaline Phosphatase (AP) Red Detection Kit), incubate for 12 minutes.
10. Apply one drop of UV Red Enhancer (part of AP Red Kit), incubate for 4 minutes.
11. Apply one drop of UV Fast Red A (part of AP Red Kit) and UV Red Naphthol (part of AP Red Kit), incubate for 8 minutes.
12. Apply one drop of UV Fast Red B (part of AP Red Kit), incubate for 8 minutes.
13. Apply one drop of Hematoxylin II (*counterstain*), incubate for 8 minutes.
14. Apply one drop of Bluing Reagent (post counterstain), incubate for 4 minutes.
15. Remove oil in Coverslip (Ultra LCS) solution by rinsing in soapy water (100 ml of DAWN Professional Liquid Concentrate Detergent (P&G Professional www.PGPRO.com) in 250 ml Tap water).

**Table 1:** Antibodies used during initial evaluation<sup>[1,2]</sup>.

Immunomarker	Antibody details	Dilution <sup>†</sup>	Pretreatment	First or second	Final color
Vimentin (in A and B)	Monoclonal, clone Vim V9, Ventana	RTU	HIER CC1 64 min	First in combination B and second in combination A	Red (Cytoplasmic) in A Brown (Cytoplasmic) in B
Cytokeratin 7 (in B)	Monoclonal, clone- SP52, Ventana	RTU	HIER CC1 36 min	Second in combination B	Red (Cytoplasmic)
Calretinin (in C and D)	Monoclonal, Clone-SP65, Ventana	RTU	HIER CC1 36 min	First in combination C and D	Brown (Nuclear [& cytoplasmic])
BerEP4 (in A and C)	Monoclonal, Clone-BER-EP4 Dako	1:80	HIER CC1 36 min	First in combination A and second in combination C	Brown (Cytoplasmic) in A Red (Cytoplasmic) in C
Cytokeratin 20 (in D)	Monoclonal, clone-SP33, Ventana	RTU	HIER CC1 36 min	Second in combination D	Red (Cytoplasmic)

HIER (Heat Induced Epitope Retrieval) Tris-EDTA buffer pH 8+0.2 at 95°C for 36 min (mild CC1)

RTU - Ready to use; min - minutes

<sup>†</sup>Antibodies were diluted with Antibody diluent reagent solution (Life Technologies)

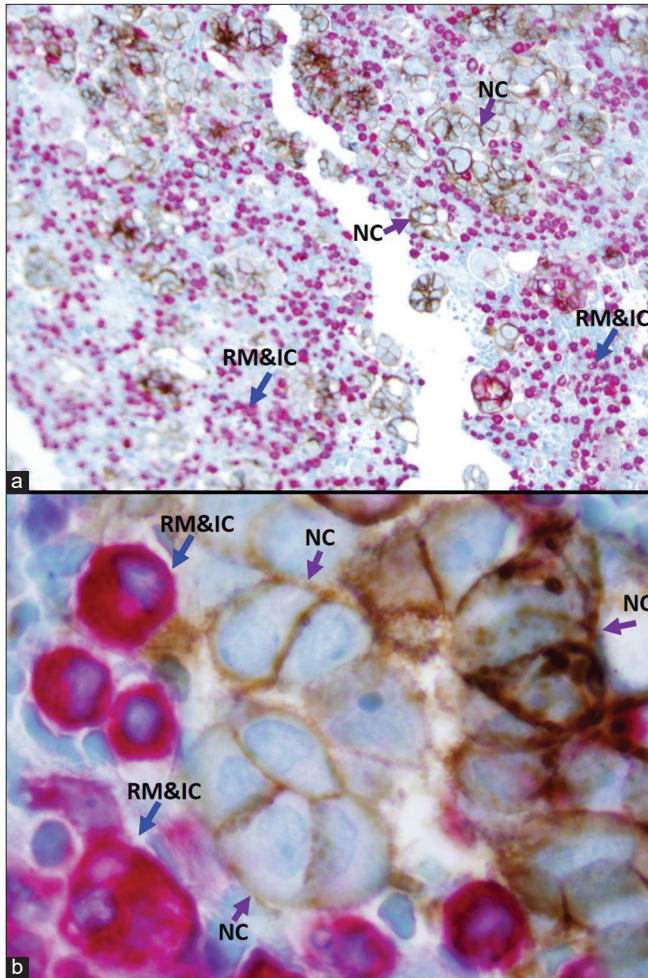
Ventana (A member of the Roche Group), 1910 Innovation Park Dr., Tucson, AZ 85755, USA

Combination A. First BerEP4 (brown) followed by vimentin (red)

Combination B. First vimentin (brown) followed by cytokeratin 7 (red)

Combination C. First calretinin (brown) followed by BerEP4 (red)

Combination D. First calretinin (brown) followed by cytokeratin 20 (red)



**Figure 1:** Dual-color immunohistochemistry with BerEP4 (brown) as first, followed by vimentin (red) immunostaining. Metastatic adenocarcinoma cells (NC) are non-immunoreactive for vimentin with immunoreactivity for BerEP4 (brown) on the background of vimentin (red) immunoreactive inflammatory cells and reactive mesothelial cells. (Pleural fluid, history of pancreatic adenocarcinoma), (a, 20X, b, 100X).

16. Dehydrate quickly in two times in 95% alcohol and two times in 100% alcohol.
17. Clear quickly with 10 dips in xylene (three times).
18. Coverslip with xylene on Tissue-Tec coverslipper.

The metastatic cells, located in relation to different components of the effusion fluid, could be evaluated further with additional immunomarkers (Table 2) in conjunction with pertinent clinical history.

Methods applying more than two immunocolors have also been reported.<sup>[35-37]</sup> Based on the reported literature, a variety of diagnostic combinations may be evaluated. As observed, a protocol-related interference in immunoreactivity pattern is a possibility. Because of this, such combinations and their immunostaining protocols should be standardized and evaluated prior to their clinical application.

## IMMUNOMARKERS COMMONLY USED IN EFFUSION IMMUNOCYTOCHEMISTRY

Some of the immunomarkers commonly applied to effusion immunocytochemistry are described below briefly in alphabetical order. The details of some of these as used in our laboratory are set out in Table 3.

### ANTIMESOTHELIAL CELL ANTIBODY

A polyclonal antibody against cytoplasmic protein of mesothelial cells was reported to be immunoreactive with all epithelioid mesotheliomas and non-immunoreactive with all of the adenocarcinomas.<sup>[38]</sup> Further studies, however, are not found in the English language literature.

### ARGINASE- 1

**Clones/synonyms:** rabbit recombinant, MSVA-511R

Arginase hydrolyses arginine into ornithine and urea. It is a binuclear manganese metalloenzyme with two isoforms: Arginase-1 and Arginase-2. Arginase-1 is present in the liver, but Arginase-2 is also found in renal and other tissues. Because of this, Arginase-1 immunoreactivity has been evaluated as hepatocytic immunomarker.

It is observed that Arginase-1 is expressed predominantly in hepatocytes and not in other tissues. It demonstrates diffuse cytoplasmic immunoreactivity in both hepatocellular neoplasms and non-neoplastic hepatocytes with patchy nuclear immunostaining. The significance of nuclear immunoreactivity is not known. Thus cytoplasmic immunoreactivity is considered significant.

Studies have reported immunoreactivity of Arginase-1 in vast majority of hepatocellular carcinomas but only rarely in non-hepatocellular tumors.<sup>[1,39-41]</sup> It represents a sensitive and specific immunomarker for benign and malignant hepatocytes with specificity of 99.6% (as compared to the specificity of 96.3% for HepPar-1).

### B72.3 (TAG-72)

**Clones/synonyms:** BRST-3, TAG-72, CC49

This is an antibody that reacts with a tumor-associated oncofetal antigen. A wide variety of adenocarcinomas, including those of lung, breast, gastrointestinal (GI) tract, ovary, pancreas, and endometrium, are immunoreactive to B72.3.<sup>[42]</sup> Melanomas, sarcomas, and leukemia/lymphomas do not show immunoreactivity for B72.3. It is negative in most non-neoplastic tissues, but normal secretory endometrium is immunoreactive.<sup>[38,43-46]</sup> The predominant immunostaining pattern with B72.3 is cytoplasmic (Figure 2,3c,d).<sup>[42]</sup>

Effusion studies show B72.3 immunoreactivity in 44–80% of adenocarcinomas,<sup>[45-47]</sup> but mostly negative or rarely positive

**Table 2:** Immunomarkers of interest for immunocytochemical evaluation of effusion fluids (in alphabetical order).

Immunomarker	Pattern	Immunoreactivity in	References
Arginase-1	Cytoplasmic	Hepatocellular Ca	[23,33,48]
B72.3	Cytoplasmic	AdCa	[38,42-52]
BERFP4	Cytoplasmic	AdCa	[44,46,52,53]
CA19-9	Cytoplasmic luminal	AdCa	[46,49,50,54-56]
E-Cadherin (HECD-1)	Membranous Cytoplasmic	AdCa	[9,50,52,57-60]
Calretinin	Nuclear (with or without cytoplasmic)	Mesothelioma Mesothelial cells	[9,50,61-64]
CD45 (LCA)	Cytoplasmic	Inflammatory cells	[65]
CD68 (PGM1)	Cytoplasmic	Histiocytes	[65]
CDX2	Nuclear	Colon	[66-69]
CEA: monoclonal (mCEA)	Cytoplasmic	AdCa	[38,43-46,56,66-69]
CEA: polyclonal (pCEA)	Bile canalicular pattern	Hepatocellular Ca	[74]
Claudin 4	Membranous	AdCa	[75-77]
Cytokeratin	Cytoplasmic	Carcinoma Mesothelioma	[44,49,50,52,78-82]
D2-40	Membranous	Mesothelioma Lymphatic endothelium, Testicular germ cell tumors	[83-85]
EMA (epithelial membrane antigen)	Membranous Cytoplasmic	Mesothelioma AdCa Lymphoma – Large cell anaplastic	[28,44,46,49,50,86-88]
GATA3	Nuclear	Breast Ca Urothelial Ca	[89-91]
HBME-1	Membranous (thick microvillus in mesothelioma)	Epithelioid mesothelioma Thyroid Ca Sarcoma Lymphoma	[50,55,87,92-94]
Mammaglobin	Cytoplasmic	Breast Ca	[95]
Mesothelin	Membranous	Mesothelioma Many AdCa	[96]
MOC-31	Cytoplasmic	AdCa	[50,52,92,97,98]
NKX3.1	Nuclear	Prostate AdCa	[99,100]
PAX2	Nuclear	Renal	[101]
PAX8	Nuclear	Mullerian, Renal, thyroid	[101-103]
Podoplanin	Membranous	Mesothelioma	[84,104,105]
SATB2	Nuclear	Colon	[76,106,107]
Thrombomodulin	Membranous	Mesothelial cells AdCa	[50,52,55,70,78,92]
TTF-1	Nuclear	AdCa – lung and thyroid	[60,108-110]

(Contd...)

**Table 2: (Continued).**

Immunomarker	Pattern	Immunoreactivity in	References
Vimentin	Cytoplasmic	Mesothelioma Sarcoma Lymphoma	[71,111-114]
WT-1	Nuclear (with or without cytoplasmic)	Mesothelioma Ovarian Ca DSRCT	[60,87,115]

AdCa: Adenocarcinoma, Ca: Carcinoma, DSRCT: Desmoplastic small round cell tumor; EMA: Epithelial membrane antigen; mCEA: monoclonal Carcinoembryonic antigen; pCEA: polyclonal Carcinoembryonic antigen; PSA: prostate-specific antigen; PSAP: prostatic-specific acid phosphatase; TTF-1: Thyroid transcription factor-1, WT-1: Wilms' tumor-1.

**Table 3: Details of antibodies (for immunocytochemistry on formalin-fixed paraffin-embedded sections of cell-blocks) (in alphabetic order).**

Immunomarker	Antibody source, type/clone, dilution, incubation time	Antigen retrieval, duration
Arginase-1	Cell Marque sp156, RTU, 32 Min	HIER pH 8.0
B72.3	BIOGENEX TAG-72,1:200,32 Min	NONE
BerEP4	Dako BER-EP4, 30 min 1:80,32 Min	HIER pH 8.0
CA19-9	CELL MARQUE 121SLE, RTU,32 Min+AMPLIFIER	HIER pH 8.0
E-Cadherin	VENTANA 36, RTU, 40 Min	HIER pH 8.0
Calretinin	VENTANA SP65, RTU, 32 Min	HIER pH 8.0
CD15 (LeuM1)	VENTANA MMA, RTU, 32 Min	HIER pH 8.0
CD45 (LCA)	CELL MARQUE 2B11&PD7/26, RTU, 16 Min	HIER pH 8.0
CD68 (PGM1)	VENTANA KP-1, RTU, 32 Min	HIER pH 8.0
CDX2	Biogenex CDX2-88, 30 min RTU, 32 Min	HIER pH 8.0
CEA: monoclonal (mCEA)	Dako 11-7, 1:100, 16 Min	HIER pH 8.0
CEA: polyclonal (pCEA)	CELL MARQUE, RTU, 32 Min	HIER pH 8.0
Claudin 4	Zymed, monoclonal 1:100,	30 Min in a retrieval solution (Target Retrieval Solution cat S1699; DAKO, Glostrup, Denmark) in a microwave oven
Cytokeratin	CELL MARQUE AE1/AE3, 1:100, 20 Min	PROTEASE 1, 4 Min
D2-40	PODOPLANIN, CELL MARQUE, D2-40, RTU, 32 Min	HIER pH 8.0
EMA (epithelial membrane antigen)	VENTANA E29, RTU, 32 Min	HIER pH 8.0
GATA3	VENTANA, L50-823, RTU, 32 Min	HIER pH 8.0
HBME-1	VENTANA HBME-1, RTU, 32 Min	HIER pH 8.0
Mammaglobin	CELL MARQUE, 31A5, RTU, 32 Min	HIER pH 8.0
Mesothelin	LEICA, 5B2, 1:20, 44 Min	HIER pH 6.0
MOC-31	CELL MARQUE 31A5, RTU, 32 Min	HIER pH 8.0
NKX3.1	CELL MARQUE, EP356, RTU, 60 Min	HIER pH 8.0

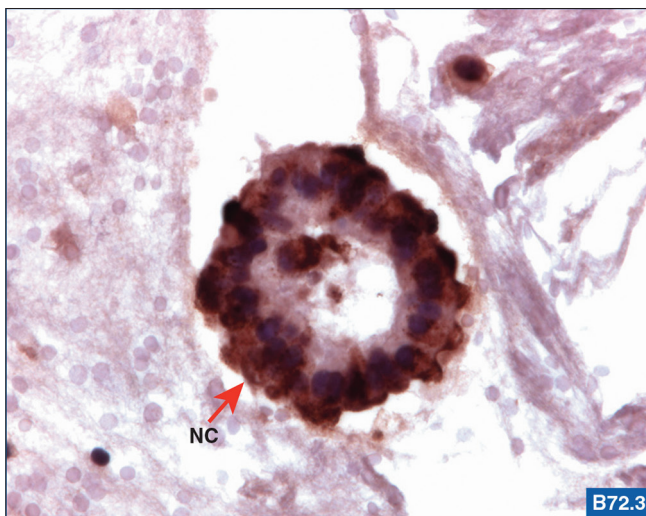
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**Table 3:** (Continued).

Immunomarker	Antibody source, type/clone, dilution, incubation time	Antigen retrieval, duration
PAX2	CELL MARQUE, EP235, RTU, 32 Min	HIER pH 8.0
PAX8	CELL MARQUE, MRQ-50, RTU, 32 Min	HIER pH 8.0
Podoplanin	D2-40, CELL MARQUE, D2-40, RTU, 32 Min	HIER pH 8.0
SATB2	CELL MARQUE, EP281, RTU, 32 Min	HIER pH 8.0
Thrombomodulin	CELL MARQUE 1009, RTU, 32 Min	HIER pH 8.0
TTF-1	VENTANA 8G7G3/1, RTU, 32 Min	HIER pH 8.0
Vimentin	VENTANA V9, RTU, 16 Min	HIER pH 8.0
WT-1	CELL MARQUE 6F-H2, RTU, 32 Min+AMPLIFIER	HIER pH 8.0

RTU, Ready to use; Min, minutes; HIER (Heat Induced Epitope Retrieval) Tris-EDTA buffer pH 8+0.2 at 95°C for 36 min (mild CC1)



**Figure 2:** B72.3 immunoreactivity pattern (metastatic mammary adenocarcinoma, pleural fluid). Metastatic adenocarcinoma cells (red arrow NC) show a cytoplasmic immunoreactivity pattern. [a, Immunostained cell-block section (a, 40X).]

(<10%) in malignant mesotheliomas and reactive mesothelial cells.<sup>[38,45,46]</sup> These results are comparable to those with surgical pathology tissue specimens.<sup>[49-52]</sup>

### **BerEP4 (HUMAN EPITHELIAL ANTIGEN)**

**Clones/synonyms:** BER-EP4 Dako

This antibody reacts with two glycoproteins along the cell surface and in the cytoplasm of epithelial cells<sup>[44-46,116]</sup> and does not show immunoreactivity with mesothelial cells, and nerve, glial, muscle, lymphoid, or mesenchymal tissue.<sup>[116]</sup> Compared to all other antiepithelial antibodies, only rare cases of epithelioid mesothelioma may show immunoreactivity with BerEP4. The immunoreactivity pattern is predominantly

membranous (Figure 4). In our experience BerEP4 is more sensitive than B72.3 (see Figure 3).

In effusion studies, BerEP4 typically shows negative immunostaining in epithelioid mesothelioma and reactive mesothelial cells. It shows immunoreactivity to adenocarcinoma cells in 32–96% of cases.<sup>[45,46,53,86]</sup> A large study on effusions reported 83% immunoreactivity in adenocarcinoma—93% ovarian, 88% GI tract, 81% lung, and 73% breast.<sup>[53]</sup> Other studies have reported some overlap of immunoreactivity with focal immunostaining in 26% of epithelioid mesothelioma and strong–diffuse immunostaining in 92% adenocarcinomas.<sup>[52]</sup> Strong–diffuse immunostaining with BerEP4 points to adenocarcinoma rather than malignant mesothelioma.

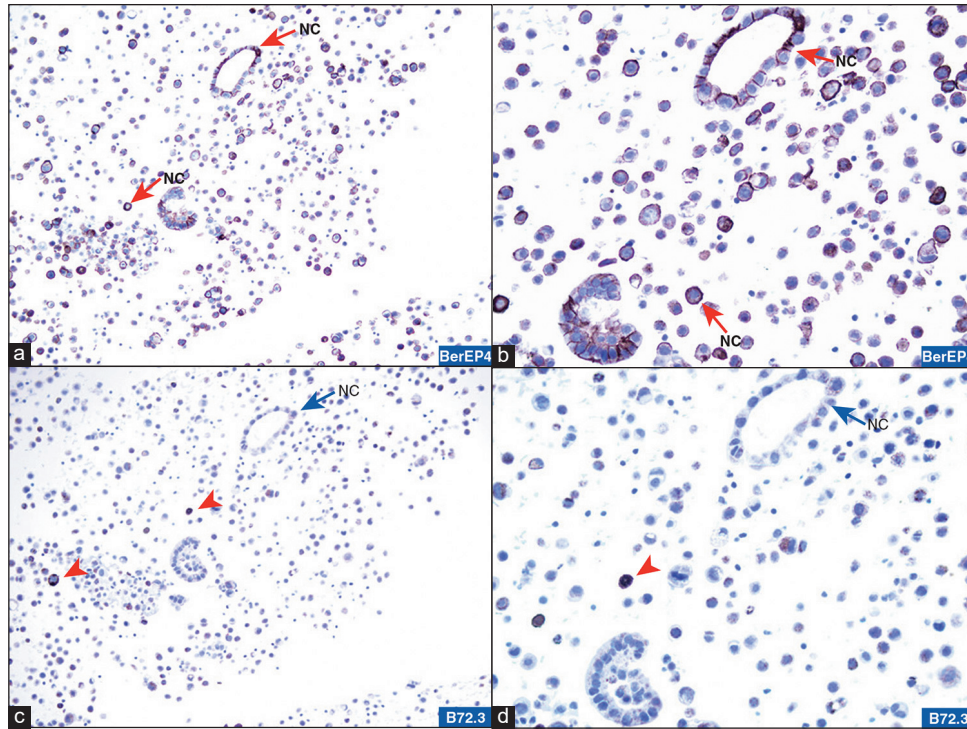
### **CA 19-9 (Cancer Antigen 19-9)**

**Clones/synonyms:** 1116NS 19-9

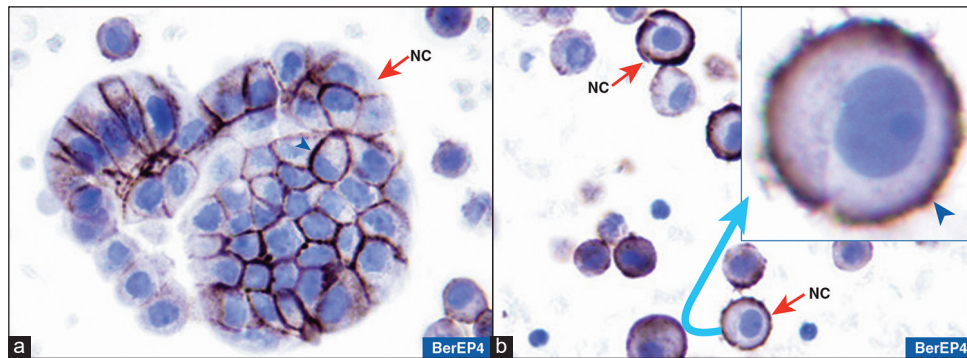
Carbohydrate antigen 19-9 (CA 19-9), a determinant (sialylated lacto-N-fucopentaose 119) of a circulating oligosaccharide antigen is biochemically related to the Lewis (a) blood group substance.<sup>[54]</sup> A monoclonal antibody BG8 is raised against lung carcinoma—SK-LU-3.<sup>[50,117]</sup> It recognizes the blood group antigen—Lewis (y). The immunostaining pattern is cytoplasmic and luminal. Cells of most pancreatic, gastric, colonic, and gall bladder adenocarcinomas are immunoreactive.<sup>[46,55,56,60,70,118]</sup> About 50% of ovarian carcinomas and 35% of mucoepidermoid carcinomas of salivary gland are immunoreactive.<sup>[118]</sup>

Non-neoplastic columnar epithelial cells lining the ducts of pancreas, stomach, liver, gallbladder, and bronchi may also show immunoreactivity for CA 19.9.<sup>[49,56,118]</sup>

Studies on surgical pathology specimens have reported CA19.9 immunoreactivity in 39–84% of adenocarcinomas and only rarely in malignant mesotheliomas.<sup>[57,118]</sup> Studies



**Figure 3:** Comparison of immunoreactivity with BerEP4 and B72.3 (metastatic mammary adenocarcinoma, pleural fluid). (a,b) Most of the adenocarcinoma cells (red arrows NC) show strong membranous BerEP4 immunoreactivity. (c,d) Compared to this, B72.3 immunoreactivity is absent in most of these neoplastic cells (blue arrows NC) with immunoreactivity in rare neoplastic cells (arrowheads). [a–d, Immunostained cell-block section (a,c, 20X; b,d, 40X).]



**Figure 4:** BerEP4 immunoreactivity pattern (metastatic mammary adenocarcinoma, pleural fluid). (a) The neoplastic cells in proliferation spheres (red arrow NC) show membranous immunostaining (arrowhead in a) with a honeycomb-like pattern. (b) Solitary adenocarcinoma cells (red arrow NC) show a membranous immunostaining pattern along the cell membrane (arrowhead in inset). [a,b, Immunostained cell-block section (a,b, 40X).]

with effusion fluids have reported comparable results with immunoreactivity in 49–86% of adenocarcinomas and rarely in malignant mesothelioma.<sup>[46,55]</sup>

Thus, positive immunostaining with CA19-9 helps to rule out malignant mesothelioma. However, for the differential diagnosis of lung carcinoma and malignant mesothelioma, CA19.9 will not be of great help, because, similar to mesotheliomas, lung carcinomas are also usually non-immunoreactive.<sup>[54,55]</sup>

### E-CADHERIN (HECD-1, EPITHELIAL CADHERIN, UVOMORULIN)

**Clones/synonyms:** 36B5, 4A2 C7, 5H9, 67A4, CDH1, CLONE36, E9, ECCD-2, ECH-6, HECD-1, SC-8426

E-cadherin is a member of transmembrane glycoproteins responsible for calcium-dependent intercellular adhesion.<sup>[57,119]</sup> It is expressed along the cell surfaces of non-neoplastic epithelial cells and their neoplastic counterparts

by a membranous immunostaining pattern in conjunction with a variable cytoplasmic pattern.<sup>[57,119]</sup> Tissue studies have reported immunoreactivity in 81–93% of adenocarcinomas, with typically non-immunoreactive results in almost all of the malignant mesotheliomas.<sup>[58,60]</sup> This pattern may assist in distinguishing between reactive and neoplastic mesothelial cells from carcinoma cells in effusions.<sup>[9,52,57,58,60]</sup>

However, effusion studies are not as conclusive, with variable results, in part due to the variation in the antibody clones used.<sup>[50,60]</sup> One study reported E-cadherin immunoreactivity in 97% of adenocarcinomas, 46% of malignant mesotheliomas, and 14% of reactive mesothelial cells.<sup>[57]</sup> Another study on cell-block sections of effusions observed non-immunoreactivity in all malignant mesotheliomas, with immunoreactivity in all adenocarcinomas.<sup>[58]</sup>

This is further complicated due to entirely different results from immunocytochemistry performed on smears. In a study on PAP-stained smears, 100% of malignant mesotheliomas and 87% of adenocarcinomas were immunoreactive, but all the reactive effusions were non-immunoreactive.<sup>[9]</sup> An additional study with ThinPrep preparations reported that in most of the cases carcinoma cells demonstrated a membranous immunostaining pattern but only one benign case showed immunoreactivity.<sup>[59]</sup> As highlighted previously, apart from the differences in the type of antibodies used, the processing of specimens by a variety of methods may also have contributed to contradicting and confusing results.

## N-CADHERIN

**Clones/synonyms:** 13A9, 3B9, GC-4

N-cadherin is associated with the developing skeletal muscle, cardiac muscle, nerve cells, and mesothelial cells.<sup>[52]</sup> It is expressed by both neoplastic and non-neoplastic mesothelial cells.<sup>[58]</sup> A study evaluating tissues and effusion fluids reported N-cadherin immunoreactivity in 92% of malignant mesotheliomas, with non-immunoreactivity in all adenocarcinomas.<sup>[44]</sup> However, other studies could not confirm this and showed low sensitivity and specificity for malignant mesotheliomas.<sup>[50,86]</sup> One of the studies on effusion specimens reported N-cadherin immunoreactivity to reactive mesothelial cells in 77% of effusions, along with immunoreactivity to 35% of malignant mesotheliomas and 48% of adenocarcinomas.<sup>[57]</sup> Thus, N-cadherin immunostaining does not have a significant role in immunocytochemical evaluation of effusions.

## CALRETININ AB1 49, DC8

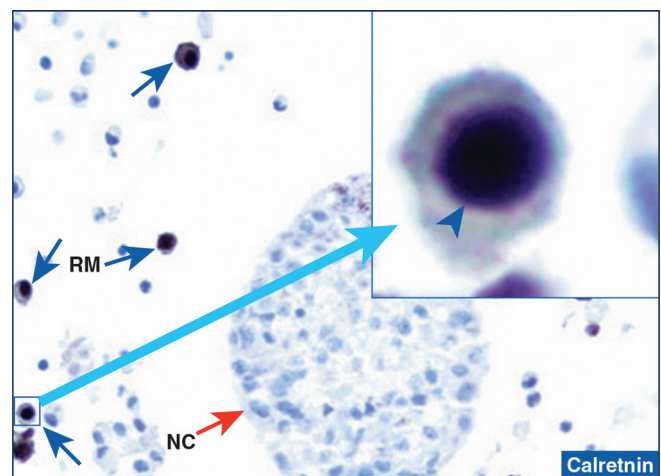
**Clones/synonyms:** Dak Calret1

Calretinin is a neuron-specific calcium-binding protein. It is strongly expressed in neural tissues and certain non-neural cell types such as mesothelial cells.<sup>[9,61,78,87,120-123]</sup> Antibodies to calretinin show strong nuclear (and cytoplasmic)

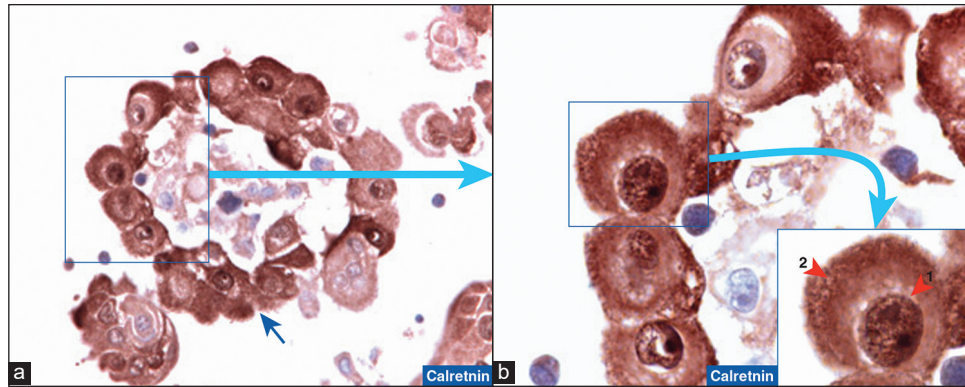
immunoreactivity in benign and neoplastic mesothelial cells. The cytoplasmic and nuclear immunostaining has been described as a ‘fried-egg’ appearance (Figures 5, 6).<sup>[62]</sup> Adenocarcinomas are usually non-immunoreactive or show weak cytoplasmic immunoreactivity in rare cells.<sup>[61,78,122,123]</sup>

Similar to other immunomarkers, different types and sources of antibodies with variable processing protocols may lead to inconsistent results. Some of the studies used polyclonal antibodies from diverse sources.<sup>[50,52,71]</sup> A few studies evaluating this immunomarker concluded calretinin to be unreliable.<sup>[58,87,92]</sup> However, with the advent of purified and monoclonal antibodies, calretinin immunostaining has demonstrated better results in distinguishing between malignant mesothelioma and adenocarcinoma in histologic and cytologic preparations.<sup>[52,121,124]</sup> A recent study evaluating polyclonal and monoclonal calretinin antibodies demonstrated similar results.<sup>[63]</sup>

Studies on tissue have reported immunoreactivity in 92–100% of malignant mesotheliomas, with only the rare adenocarcinoma demonstrating nuclear immunoreactivity.<sup>[61,78]</sup> Many studies with cell-blocks, Cytospins, and de-stained PAP-stained smears of effusions have demonstrated encouraging results with reactive and neoplastic mesothelial cells with calretinin immunoreactivity (nuclear and cytoplasmic) in 88–100% of mesotheliomas and 80–100% of reactive effusions.<sup>[9,62,64,123,125]</sup> We find calretinin to be an excellent immunomarker for evaluation of effusion cytopathology if properly applied and interpreted. We use monoclonal antibody for calretinin (Clone Dak Calret 1, Dako Corporation, Carpinteria, CA; dilution: 1:400, for 30 minutes at room temperature, after heatinduced epitope retrieval in 10 mmol/L



**Figure 5:** Calretinin immunoreactivity pattern (pleural fluid). Reactive mesothelial cells (blue arrows). The effusion also contains metastatic mammary carcinoma cells (red arrow NC). The diagnostic immunoreactivity pattern is nuclear (arrowhead in inset), which is usually associated with concurrent cytoplasmic immunoreactivity (blue arrow RM). Cytoplasmic immunoreactivity alone should be considered non-specific for interpretation of mesothelial cells with calretinin. [Immunostained cell-block section (40X).]



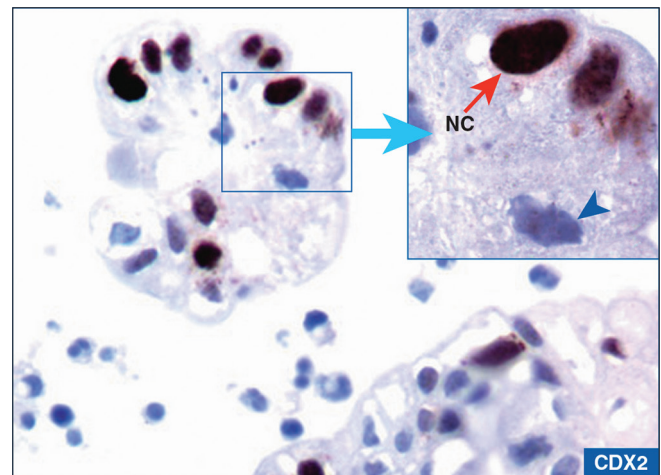
**Figure 6:** *Calretinin immunoreactivity pattern* (epithelioid mesothelioma, pleural fluid). Mesothelioma cells (arrow in a) show *nuclear* (arrowhead 1) immunoreactivity, usually with cytoplasmic immunostaining (arrowhead 2), imparting the so-called 'fried-egg' appearance. In the absence of concurrent nuclear immunostaining, cytoplasmic immunoreactivity alone should be considered non-specific for mesothelial interpretation. [a,b, Immunostained cell-block section (a, 40X; b, 100X zoomed).]

citrate buffer, pH 6.0, for 35 minutes with 20 minutes cooling time at room temperature).<sup>[124]</sup> As a part of an optimal immunopanel, calretinin is extremely valuable for distinguishing singly scattered cells of adenocarcinomas from reactive mesothelial cells.

## CDX2

**Clones/synonyms:** 7C7/D4, CDX-2-88

CDX2 is a homeobox gene that encodes an intestinespecific transcription factor alpha-methylacyl-CoA racemase (AMACR/P504S). It has fairly restricted expression in selective neoplasms. It is associated with an adenomatous polyposis coli (APC) mutation and activation of the Wnt pathway.<sup>[66]</sup> CDX2 immunostaining may be useful in discriminating between colorectal carcinomas and other adenocarcinomas of non-gastrointestinal origin.<sup>[67,69]</sup> With diagnostic nuclear immunostaining pattern, CDX2 has shown promising results (Figure 7) with effusions (personal experience).



**Figure 7:** *CDX2 immunoreactivity pattern* (metastatic colonic adenocarcinoma, peritoneal fluid). The adenocarcinoma cells show nuclear immunoreactivity (arrow NC). Compare with non-immunoreactive nuclei with blue hematoxylin counterstain (arrowhead). [Immunostained cell-block section (40X).]

## CEA CARCINOEMBRYONIC ANTIGEN)

**Clones/synonyms:** 11-7, 12.140.10, A115, A5B7, CEJ065, CEMO10, COL-1, D14, IL-7, M773, PARLM1, T84.66, TF3H8-1, ZC23

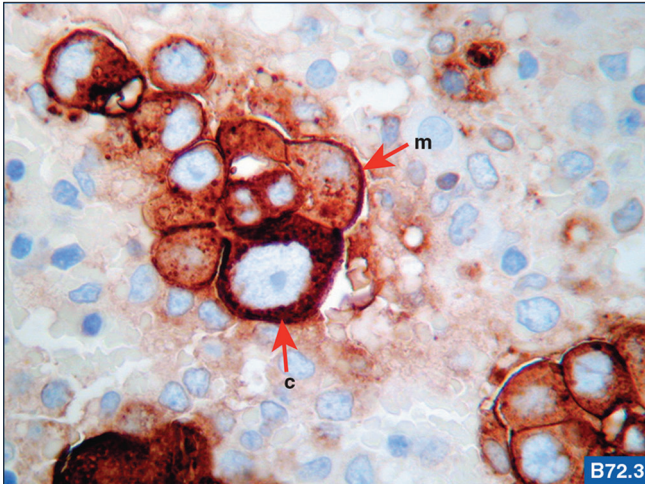
Immunoreactivity with the monoclonal antibodies to CEA is relatively specific for adenocarcinoma. It is typically nonimmunoreactive in reactive and malignant mesothelioma cells.<sup>[45,74]</sup> With the exception of granulocytes, non-malignant tissues do not show significant immunoreactivity.<sup>[74]</sup> The immunostaining associated with colorectal and other carcinomas of lung, breast, and stomach<sup>[38,43-46,56,72]</sup> is cytoplasmic and membranous (Figure 8).

Since the immunoreactivity varies considerably with the type of antibody used,<sup>[45]</sup> it is critical to select the proper CEA antibody when ordering the immunostain: pCEA (polyclonal antibodies to CEA) are more sensitive, but they demonstrate

cross-reactivity with CEA-related substances and show strong immunostaining of neutrophils and macrophages.<sup>[45,52]</sup> Although this cross-reactivity phenomenon is desirable when evaluating bile canaliculi in hepatocellular carcinoma, it may be misleading in the differential diagnosis of effusions. This cross-reactivity by pCEA may have resulted in the reports documenting immunoreactivity for CEA in mesothelial cells; CEA immunoreactivity is usually not observed in malignant mesothelioma with mCEA.<sup>[71,72]</sup>

Tissue studies report mCEA immunoreactivity in 85–94% of adenocarcinomas and virtually negative immunoreactivity in malignant mesothelioma.<sup>[49-51]</sup> Non-mucinous ovarian adenocarcinomas are usually non-immunoreactive for CEA.<sup>[49,50]</sup>

Reactive mesothelial cells and malignant mesothelioma cells in effusions are typically non-immunoreactive for CEA,<sup>[38,45,46,72]</sup>



**Figure 8:** Monoclonal CEA (mCEA) immunoreactivity pattern (metastatic ovarian carcinoma, peritoneal fluid). Metastatic adenocarcinoma cells show cytoplasmic (c) and membranous (m) immunostaining. [Immunostained cell-block section (100X).]

but immunoreactivity rates for adenocarcinomas are 21–79%. This rate for effusions is lower than that reported for tissue studies. This discrepancy may be due to the type of antibodies used in different studies since monoclonal antibodies are less sensitive than polyclonal antibodies.<sup>[45,46,56,7]</sup>

Studies has reported that pCEA (polyclonal CEA) antibody can be applied for evaluation of hepatocellular lesions. It demonstrates canalicular immunostaining pattern in hepatocytes (both neoplastic and non-neoplastic) in Paraffin-embedded cell-blocks (but may show diffuse cytoplasmic immunostaining in non-hepatocytic lesions). mCEA antibody has no value in the differential diagnosis of hepatocellular carcinoma.

#### CLAUDIN-4

**Clones/synonyms:** clone 3E2C1, Zymed

A tight junction protein, Claudin-4 (Clostridium perfringens enterotoxin receptor), is encoded by CLDN4 gene. It is considered distinguishing immunomarker for differential diagnosis of mesothelioma and adenocarcinoma. However, it may not distinguish between squamous cell carcinoma from mesothelioma.<sup>[126]</sup> Most of the metastatic adenocarcinoma were immunoreactive for claudin-4 in 95.6% cases as compared to 86.2% for Ber-Ep4.<sup>[75]</sup> Thus Claudin-4 is better immunomarker as compared Ber-Ep4. In other study all 64 cases of mesothelioma were non-immunoreactive for claudin-4 as compared to immunoreactivity in 83 of 84 adenocarcinoma (99%). Immunoreactivity in most of the adenocarcinomas showed strong diffuse membranous immunostaining with moderate intensity in a few cases. The study concluded that Claudin-4 demonstrated high sensitivity and specificity for distinguishing adenocarcinoma from malignant mesothelioma.<sup>[76,77,127]</sup>

## CYTOKERATINS

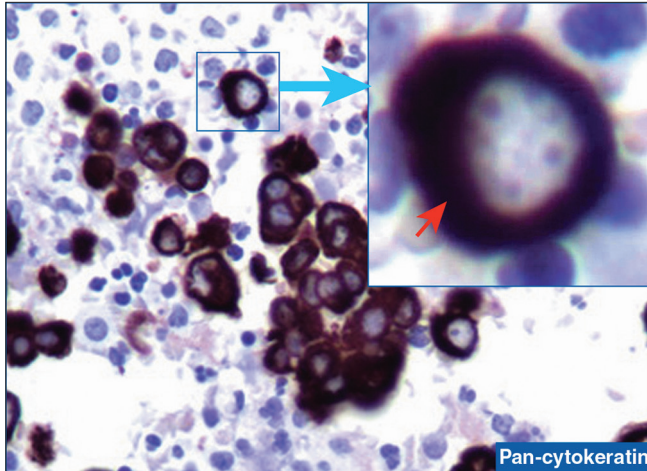
These intermediate-sized monofilaments form part of the cytoskeleton. They are present in the cytoplasm and along the cytoplasmic membrane in nearly all true epithelial structures (both neoplastic and non-neoplastic).<sup>[44,49,79-81]</sup>

Broadly, they are divided into low and high molecular weight cytokeratins. • **Non-neoplastic mesothelial cells and meso-thelioma cells are immunoreactive for both low and high molecular weight cytokeratins. Adenocarcinomas are usually immunoreactive for low molecular weight keratin.**

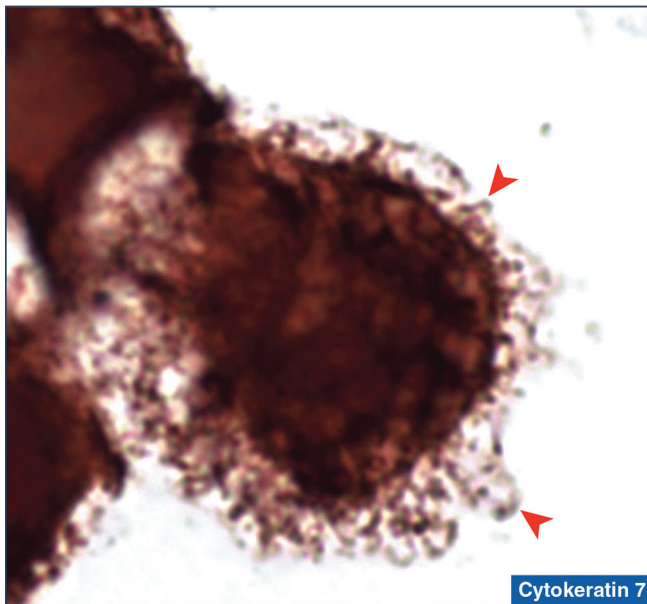
<sup>[45]</sup> Since the type of cytokeratin expressed shows significant overlap (such as the immunoreactivity of spindle cell type of malignant mesothelioma for only low molecular weight cytokeratin), it is not a reliable means for distinguishing mesotheliomas from adenocarcinomas.<sup>[49,70]</sup>

A cocktail of antibodies immunoreactive to most of the cytokeratins or an antibody immunoreactive to the common epitope present in all cytokeratins may be included in a panel to rule out other neoplasms such as lymphoma and melanoma. Cytokeratin immunoreactivity would also establish immunointegrity of the cells under scrutiny.<sup>[49,81]</sup> A murine monoclonal antibody, mAB Lu-5, has been reported as a formaldehyde-resistant cytokeratin marker. The epitope is located on most of the cytokeratin polypeptides of both the acidic (type I) and basic (type II) subfamilies. It has a cytoplasmic immunostaining pattern and has been recommended as a first-order 'pan-epithelial marker' to distinguish epithelial neoplasms from other non-epithelial neoplasms such as sarcoma, lymphoma, and melanoma.<sup>[128,129]</sup> In our experience, however, some neoplasms that were otherwise immunoreactive for one of the cytokeratin antibodies did not show immunoreactivity with Lu-5. A cocktail of cytokeratin AE1/AE3 and CAM5.2 shows better results (personal experience) (SFigure 9).

Other specific types of cytokeratins, such as CK 5/6, CK 7, and CK 20, may have a role in evaluation of effusions. Studies have reported the application of CK 5/6 for distinguishing epithelioid mesothelioma from adenocarcinomas.<sup>[50,52,78]</sup> CK 5/6 immunoreactivity was observed in 92–100% of malignant mesotheliomas,<sup>[52,78]</sup> with only 0–14% of adenocarcinomas showing weak focal immunoreactivity.<sup>[52,78]</sup> However, it has not been widely reported for effusions. CK 7 and CK 20 may have a role in different situations, including evaluation of an unknown primary neoplasm.<sup>[82]</sup> The pattern of immunostaining with cytokeratin 7 may help to identify reactive and neoplastic mesothelial cells (Figure 10). Similarly, as compared to adenocarcinoma cells, mesothelial cells may show a concentric immunostaining pattern (better appreciated by adjusting fine focus) for pancytokeratin around the nucleus (Figure 9).



**Figure 9:** Pan-cytokeratin immunoreactivity pattern (pleural fluid). Reactive mesothelial cells with cytoplasmic immunostaining (arrow in inset). Some reactive mesothelial cells may show a concentric immunostaining pattern around the nucleus, which is better appreciated by adjusting fine focus. [Immunostained cell-block section (40X).]



**Figure 10:** Cytokeratin 7 immunoreactivity pattern (epithelioid mesothelioma, pleural fluid). Neoplastic mesothelial cells with cytoplasmic immunostaining. Note the bushy microvilli (arrowhead). [Immunostained cell-block section (100X zoomed).]

### D2-40 (SEE ALSO PODOPLANIN)

D2-40 is a novel monoclonal antibody to M2A antigen, a relative molecular mass (Mr) 40 000 O-linked sialoglycoprotein that reacts with a fixation-resistant epitope. M2A antigen expression was reported in testicular germ cell tumors<sup>[130]</sup> and lymphatic endothelium.<sup>[131]</sup> Recently, D2-40 immunoreactivity has been reported as an immunomarker for reactive and neoplastic

mesothelial cells. It has been recommended for the differential diagnosis of mesothelioma from adenocarcinoma.

The observed sensitivity was similar to calretinin but better than cytokeratin 5/6 and WT-1.<sup>[83-85]</sup> Recently, some studies have reported D240 expression in primary peritoneal serous carcinomas and ovarian tumors.<sup>[132]</sup> One of the meta-analysis, concluded that D2-40 immunostaining alone may not be sufficient to diagnose malignant melanoma.<sup>[127]</sup> Recently, it is reported that commercially available mouse monoclonal antibody D2-40 in fact recognizes human podoplanin (see below).<sup>[143-145]</sup>

### EPITHELIAL MEMBRANE ANTIGEN (EMA)

**Clones/synonyms:** 214D4, E29, GP1.4, MC5

EMA, one of the human milk fat globule membrane proteins, is associated with a wide variety of neoplastic and non-neoplastic epithelia.<sup>[38,44,45,86,104,136,137]</sup> Both mesothelial and non-mesothelial epithelial elements are immunoreactive for EMA. Various squamous and glandular epithelia and lymphoid neoplasms (i.e. anaplastic large cell lymphomas) may show membranous immunoreactivity. Mesothelial cells in effusions may show weak immunoreactivity.

EMA immunoreactivity in effusions is reported in 75–100% of epithelioid mesothelioma, 91% of adenocarcinomas, and 6% of reactive mesothelial cells.<sup>[38,45]</sup> Comparable results are reported with EMA immunoreactivity in 89.5% of epithelioid mesothelioma and 100% of adenocarcinomas in surgical pathology specimens.

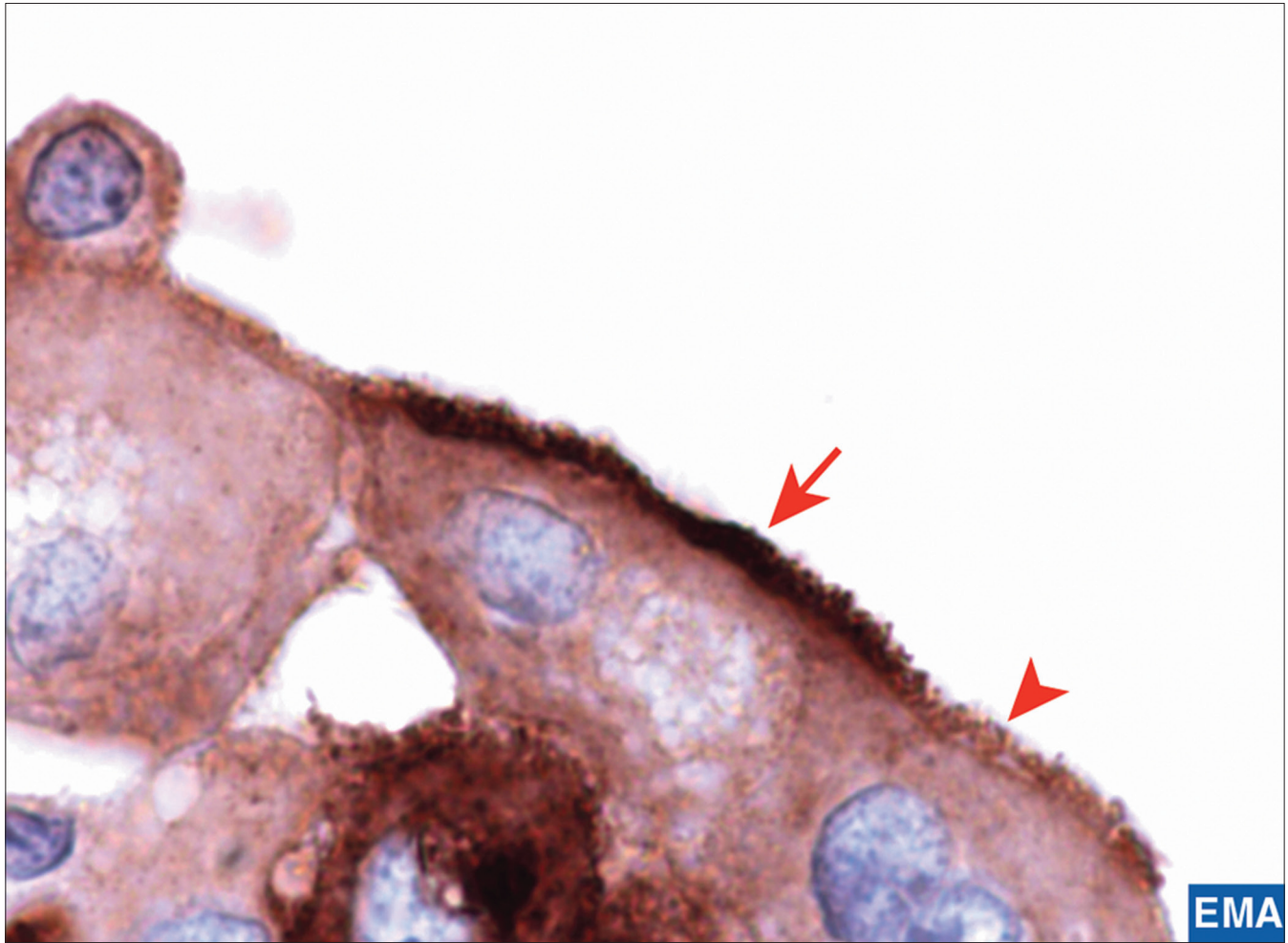
The pattern of immunostaining is the key to interpretation. Epithelioid mesothelioma shows a characteristic immunoreactivity pattern, with EMA demonstrating a ‘thick’ membranous immunostaining along the periphery of cell clusters highlighting the long microvilli (Figures 11,18a,b)<sup>[70,104]</sup> Adenocarcinoma cells are also immunoreactive for EMA, but the immunostaining pattern is usually cytoplasmic (see Figure 2a).<sup>[70,104]</sup> Due to relative difficulty in evaluating immunostaining patterns, EMA usually is not a favored addition to the panel of immunomarkers in effusions for distinguishing between epithelioid mesothelioma and adenocarcinoma.<sup>[52]</sup>

After evaluating clones E29 and Mc5 of EMA antibodies, E29 has been reported to distinguish between non-neoplastic reactive mesothelial cells and neoplastic mesothelioma cells. In this study, 75% of malignant mesotheliomas showed immunoreactivity for EMA with clone E29, but reactive mesothelial cells were non-immunoreactive in all cases.<sup>[138]</sup>

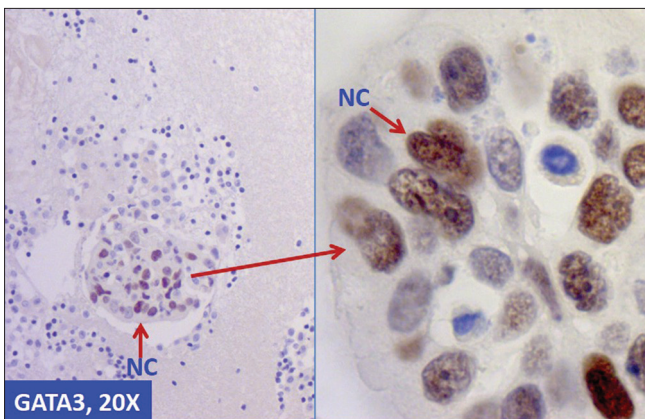
### GATA3

**Clones/synonyms:** L50-823, Cell Marque

GATA3 is one of the 6 GATA family of zinc-finger transcription factors. It is involved in cell development and



**Figure 11:** EMA immunoreactivity pattern (epithelioid mesothelioma, pleural fluid). Mesothelioma cells with membranous (arrow) and cytoplasmic immunostaining. Note the microvilli (arrowhead). [Immunostained cell-block section (100X zoomed)].



**Figure 12:** GATA 3: Metastatic adenocarcinoma mammary primary (history of infiltrating duct carcinoma of breast). Nuclear immunoreactivity for GATA 3 in metastatic carcinoma cells (NC). (Pleural fluid).

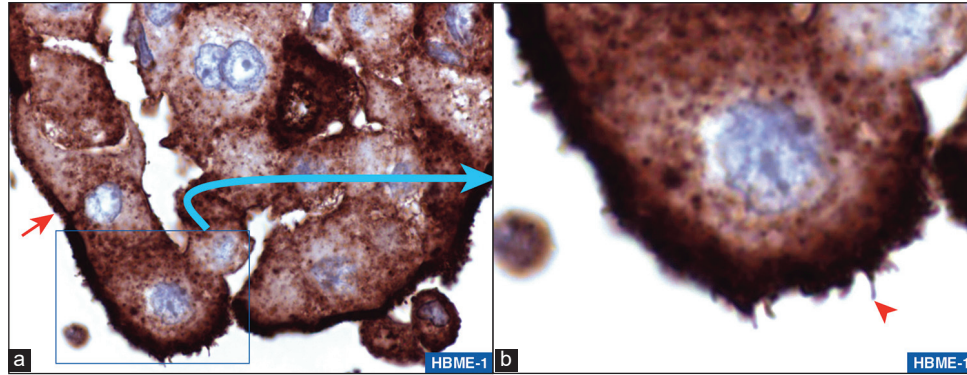
differentiation including luminal differentiation of breast epithelium, development of collecting system/urothelium,

and trophoblastic differentiation. Studies have reported GATA3 expression in neoplasms of both urothelium and breast.<sup>[91,139]</sup> It is reported to be either absent or expressed rarely in other epithelial tumors, except salivary gland and parathyroid tumors.<sup>[139]</sup>

Studies have reported diffuse, moderate to strong nuclear GATA3 immunoreactivity (Figure 12) in breast carcinomas<sup>[140]</sup> and adenocarcinomas of the urinary bladder including those with signet ring features.<sup>[89-91,139]</sup>

### HBME-1

HBME-1 immunoreactivity is observed along the cell membrane of mesothelial cells (Figures 13,18c-e).<sup>[55,87,93,94]</sup> Normal cells other than mesothelial cells are not immunoreactive for HBME-1. However, sarcomas, chordomas, and lymphomas, along with papillary and follicular carcinomas of the thyroid, show HBME-1 immunoreactivity.<sup>[50,93]</sup>



**Figure 13:** HBME-1 immunoreactivity pattern (epithelioid mesothelioma, pleural fluid). Mesothelioma cells with membranous (arrow in a) and cytoplasmic immunostaining. Note the microvilli (arrowhead in b). [a,b, Immunostained cell-block section (a, 100X; b, 100X zoomed).]

The characteristic immunoreactivity pattern with epithelioid mesothelioma is thick membranous immunostaining (see Figures 13,18c-e), but with adenocarcinomas it is usually cytoplasmic, and if membranous it is flimsy and thin (see Figure 2b).<sup>[55,94]</sup> Desmoplastic/sarcomatous variants of mesotheliomas are usually non-immunoreactive for membranous immunostaining pattern with HBME-1. This is considered to be due to the loss of microvilli on the cell surface of these variants.<sup>[93]</sup>

Many studies performed on tissue specimens have reported overlapping results in epithelioid mesothelioma and adenocarcinomas,<sup>[50,52,87,92]</sup> concluding that HBME-1 is less efficient than the other markers for definitive identification of mesothelioma.<sup>[92]</sup> Similarly, a study on effusions reported immunoreactivity in 89% of mesotheliomas and 65% of adenocarcinomas, with thick and thin membranous immunostaining patterns in both entities.<sup>[55]</sup> Thus, HBME-1 appears to be of limited application for evaluation of effusion specimens.

## IMMUNOMARKERS FOR INFLAMMATORY CELL COMPONENT

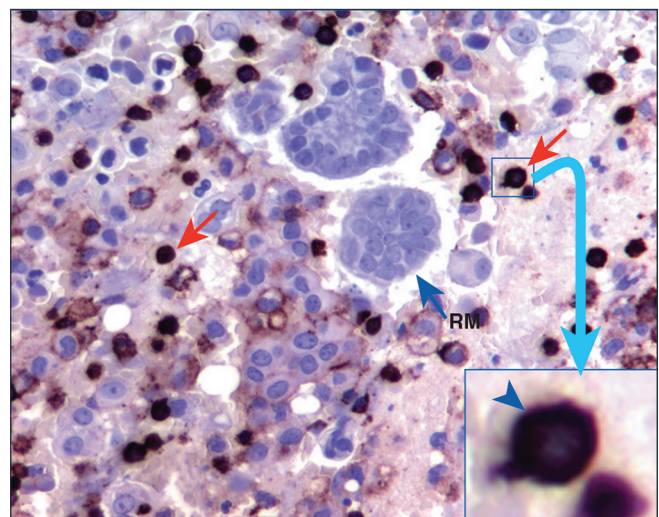
### CD68

**Clones/synonyms:** KP-1, PG-M1

### CD45

**Clones/synonyms:** 1.22/4.14, 2B11+PD7/26, 2D1, LCA, PD7, PD7/26, T29/33

LCA (leukocyte common antigen, CD45) or PGM1 (CD68) or a mixture of LCA and PGM1 may be used as an immunomarker for inflammatory cells<sup>[65]</sup> and also for lymphoma leukemia cells. Both LCA (Figure 14) and PGM1 (Figure 15) show a cytoplasmic immunoreactivity pattern. Since LCA (CD45) is the stronger antibody (see Figure 14), it does not require the antigen retrieval step. In comparison, PGM1 (CD68) needs the antigen retrieval step. To cover all inflammatory cells in the effusions for creation of a basic map



**Figure 14:** LCA (CD45) immunoreactivity pattern (pleural fluid). Reactive mesothelial cells (blue arrow RM) with chronic inflammatory cells (red arrows). The inflammatory cells show a strong cytoplasmic immunoreactivity pattern obscuring the nucleus (arrowhead in inset). [Immunostained cell-block section (40X).]

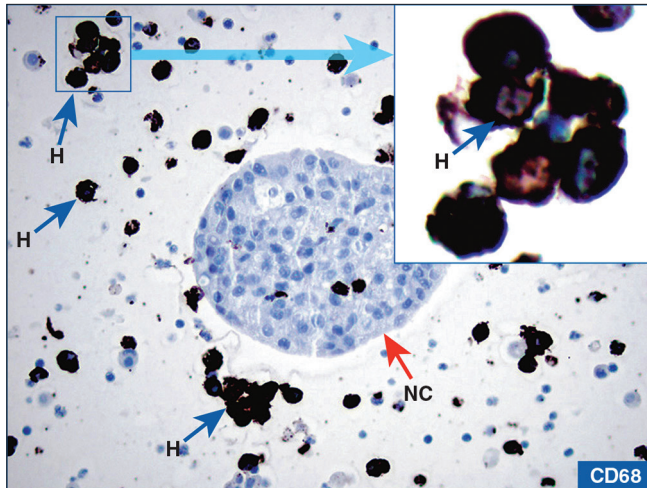
for evaluation with the SCIP approach, LCA and PGM1 may be combined. However, the titer of LCA antibody will have to be adjusted to accommodate the antigen retrieval used for PGM1. In our experience, PGM1 usually lacks the non-specific immunoreactivity frequently observed with KP1, and so we prefer PGM1 over KP1 as our CD68 antibody.

## MESOTHELIN

**Clones/synonyms:** 5B2

Mesothelin is a differentiation antigen targeted by the monoclonal antibody K1. It is a 40 kDa cell surface protein involved in cell-cell adhesion. It is strongly expressed in normal mesothelial cells, mesotheliomas, non-mucinous ovarian carcinomas, and some other cancers. With the availability of 'second-generation' antimesothelin





**Figure 15:** CD68 (PGM1) immunoreactivity pattern (metastatic mammary adenocarcinoma with proliferation spheres (red arrow NC), pleural fluid). Histiocytes show CD68 immunoreactivity (blue arrows H). In our experience, PGM1 does not show non-specific immunostaining usually associated with KP1. Inset, histiocytes (blue arrow H) with cytoplasmic immunoreactivity pattern around the nucleus. [Immunostained cell-block section (40X).]

antibodies such as the 5B2 clone, it can now be evaluated by immunocytochemistry. It is regarded as specific for mesothelial cells (and surface ovarian epithelial cells), but it is expressed in many adenocarcinomas,<sup>[96]</sup> with a membranous immunostaining pattern along the cell membrane. It does not appear to be an effective immunomarker for evaluation of effusion fluids.

### MOC-31

MOC-31 is an antibody raised by using the GLS-1 cell line of small cell carcinoma of lung. This antigen is a human cell surface glycoprotein related to an epithelial antigen present on the most neoplastic and non-neoplastic epithelia.<sup>[52]</sup> It has demonstrated immunoreactivity with the majority of adenocarcinomas, but only rarely with mesotheliomas and reactive mesothelium.<sup>[50,52,92,141]</sup>

MOC-31 has thus been recognized as one of the 'negative' mesothelioma markers for the differential diagnosis of adenocarcinoma and mesothelioma. It reacts with a 38 kDa protein associated with a wide variety of adenocarcinomas; 62 of 63 adenocarcinomas of various origins were positive and five epithelioid mesotheliomas were all negative for MOC31.<sup>[97]</sup> Many other studies have also demonstrated the role of MOC-31 in the differential diagnosis of mesothelioma.<sup>[50,52,92,97,141]</sup> The cytoplasmic and membranous immunoreactivity pattern of MOC-31 is usually strong and diffuse with the adenocarcinomas (Figure 16), in contrast to a weak and focal immunoreactivity in mesotheliomas. MOC-31 has been described as a useful immunomarker



**Figure 16:** MOC-31 immunoreactivity pattern (metastatic mammary carcinoma, pleural fluid). The adenocarcinoma cells show predominantly membranous (m) with cytoplasmic (c) immunoreactivity. [Immunostained cell-block section (100X).]

for distinguishing between epithelioid mesothelioma and pulmonary adenocarcinoma involving the pleura.

### NKX3.1

NKX3.1 protein is product of NKX3.1 gene located on chromosome 8p21.2. It functions as a haploinsufficient tumor suppressor gene. It is an androgen-regulated homeodomain gene expressed predominantly in prostate epithelium.<sup>[99]</sup>

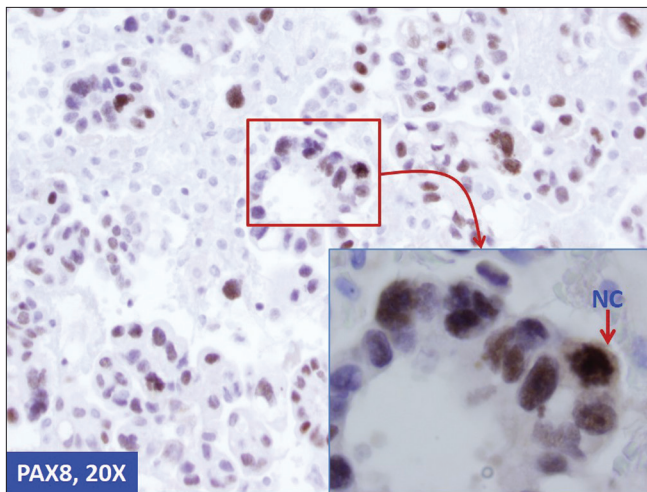
Studies have reported NKX3.1 immunostaining to be highly sensitive and specific for prostatic adenocarcinoma as compared to urothelial carcinoma.<sup>[100]</sup> The study evaluated nuclear NKX3.1 immunoreactivity in adenocarcinomas of the urinary bladder, and cancers from various sites including the breast, colon, salivary gland, stomach, pancreas, thyroid, and central nervous system, adrenal cortex, kidney, liver, lung, and testis. With sensitivity of 98.6% (68/69 cases positive) for metastatic prostatic adenocarcinomas (as compared to 94.2% (65/69 for PSA and 98.6% for PSAP) and specificity of 99.7% (1/349 non-prostatic tumor, 1 case of lobular carcinoma of breast was positive) is a valuable adjunct to distinguish prostatic primary from other metastatic carcinomas.<sup>[99,100]</sup>

### PAX2<sup>[101,142,143]</sup> and PAX8<sup>[101-103]</sup>

PAX2 and PAX8 belong to the paired box gene (PAX) family (with 9 members PAX1 through PAX9) of transcription factors which play crucial role in renal and thyroid development. Recently, expression of both PAX2 and PAX8 has been reported in malignant neoplasms of Mullerian and renal origin. Neither PAX2 nor PAX8 immunoreactivity is noted in metastatic adenocarcinomas from breast, lung,

gastrointestinal tract, and pancreaticobiliary primaries.<sup>[142]</sup> Nuclear immunoreactivity for PAX8 (Figure 17) is reported in clear cell, papillary, thymic tumors, and chromophobe renal cell carcinoma (RCC).<sup>[102,142]</sup> Direct comparison of PAX2 and PAX8 demonstrated that PAX8 (88 of 99 cases; 89%) is a more sensitive for metastatic RCC than PAX2 (75 of 99 cases; 76%).<sup>[143]</sup> However, a small subset of metastatic RCCs express only PAX2 and so both PAX8 and PAX2 should be included in a panel.<sup>[143]</sup>

However, PAX2 and PAX8 immunoreactivity is not restricted only to RCC.<sup>[142]</sup> They are expressed in Mullerian clear cell carcinoma, nephrogenic adenoma, and parathyroid parenchymal lesions. PAX8 but not PAX2 is expressed in thyroid follicular neoplasms and well differentiated neuroendocrine tumors.<sup>[143]</sup> PAX8 immunoreactivity in lung cancers regardless of tumor subtype is rare.<sup>[103]</sup>



**Figure 17:** PAX 8: Metastatic adenocarcinoma Mullerian primary (patient had ovarian mass). Nuclear immunoreactivity for PAX8 in metastatic carcinoma cells (NC). (Ascitic fluid).

### SATB2

SATB2 is AT-rich sequence DNA-binding protein with 733 amino acids demonstrating significant degree of evolutionary conservation. The difference between mouse and human protein is only three amino acids. It binds nuclear matrix attachment regions and is involved in chromatin remodeling and transcriptional regulation.<sup>[144]</sup>

SATB2 expression is feature of glandular cells of the distal gastrointestinal tract. This feature is preserved in colorectal cancers. Studies reported SATB2 immunoreactivity as sensitive and highly specific immunomarker for colorectal cancer. SATB2 immunoreactivity is reported in more than 95% of colorectal carcinoma.<sup>[106,107,144]</sup>

### PODOPLANIN (see also D2-40)

Podoplanin is a mucin-type glycoprotein that is reported to be specific for the endothelium of lymphatics. It was also

observed to be expressed in reactive mesothelial cells. Later it was evaluated in mesothelioma in tissue sections. All five malignant mesotheliomas demonstrated podoplanin immunoreactivity along the cell membrane. All other 118 neoplasms (including 93 adenocarcinomas, 4 squamous cell carcinomas, 6 gastrointestinal stromal tumors, and 5 endocrine tumors) were negative for podoplanin.<sup>[105]</sup> It was concluded that podoplanin has the potential to be an excellent tumor marker with high specificity and sensitivity. Other studies on tissue sections have reported encouraging results.<sup>[84,85]</sup> However, strong immunoreactivity of podoplanin in granulosa cells of normal ovarian follicles and in cells of ovarian dysgerminoma and granulosa cell tumors has been reported. Although it is primarily absent from normal human epidermis, strong podoplanin immunoreactivity is reported in 22 of 28 squamous cell carcinomas.<sup>[111]</sup> The mouse monoclonal antibody, D2-40, recognizes human podoplanin.<sup>[133-135]</sup> The role of these immunomarkers as an additional positive mesothelial cell marker is evolving.

### THROMBOMODULIN (CD141)

**Clones/synonyms:**1009

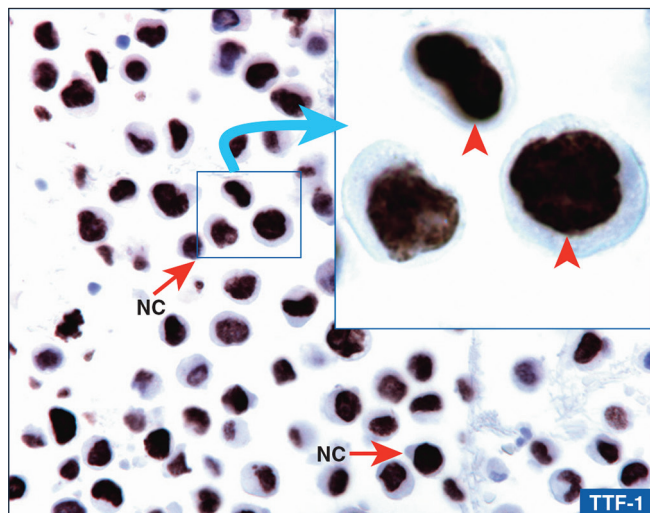
Thrombomodulin is a glycoprotein with a membranous immunostaining pattern along the cell membrane.<sup>[50]</sup> Cytoplasmic immunostaining, if present, is most likely nonspecific. It is normally expressed in endothelial and mesothelial cells along with megakaryocytes, mesangial cells, some squamous epithelial cells, and other epithelial cells including synovial cells.<sup>[70]</sup>

Tissue studies of thrombomodulin showed variable immunoreactivity in 43–90% of malignant mesotheliomas with relatively high specificity.<sup>[52,78,92]</sup> However, an effusion study demonstrated immunoreactivity in 67% of malignant mesotheliomas and 53% of adenocarcinomas with nondiscriminatory results for their differential diagnosis.<sup>[55]</sup>

### THYROID TRANSCRIPTION FACTOR-1 (TTF-1)

**Clones/synonyms:** 8G7G3/1, SC-13040, SPT-24

TTF-1 is a tissue-specific transcription factor expressed in normal lung and thyroid tissue.<sup>[108]</sup> Additional studies<sup>[109,110]</sup> have demonstrated a high specificity of TTF-1 for lung and thyroid carcinomas with a nuclear immunoreactivity pattern (Figure 18). It has demonstrated its role in distinguishing between mesotheliomas and pulmonary adenocarcinomas.<sup>[60]</sup> Seventy-five percent of pulmonary and 100% of thyroid adenocarcinomas demonstrated immunoreactivity for TTF-1; however, all other adenocarcinomas and mesotheliomas were non-immunoreactive.<sup>[60]</sup>



**Figure 18:** *TTF-1* immunoreactivity pattern (metastatic pulmonary carcinoma, pleural fluid). The solitary adenocarcinoma cells as the predominant population (arrows NC) show nuclear immunoreactivity (arrowheads in inset). [Immunostained cell-block section (40X).]

## VIMENTIN

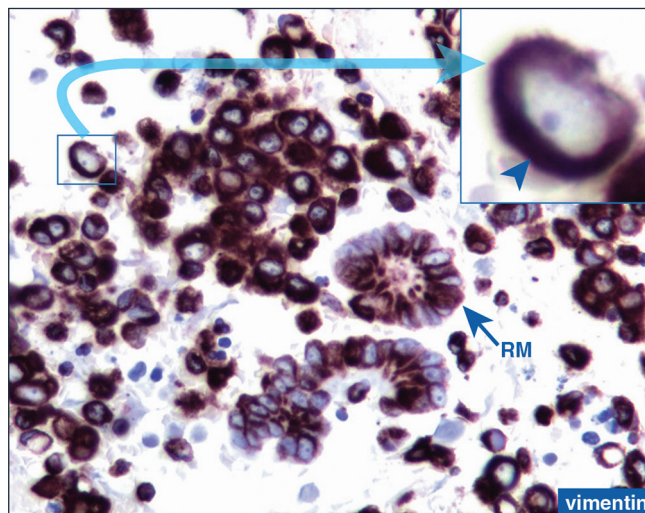
**Clones/synonyms:** 3B4, RPN1102, V10, V9, V1M-3B4

A comparative study of 11 immunohistochemical markers concluded that vimentin is one of the two best immunomarkers for distinguishing between epithelioid mesotheliomas and adenocarcinomas, with cytoplasmic immunoreactivity in mesothelial cells (Figure 19).<sup>[112]</sup> However, other studies did not find vimentin a useful immunomarker in the diagnosis of mesothelioma.<sup>[71,86,113,114]</sup> If it is understood that adenocarcinomas from some primary sites such as endometrium and kidney<sup>[22]</sup> are also immunoreactive for vimentin, it is a good immunomarker to distinguish between mesothelioma and adenocarcinoma. This highlights the significant variations in approach to immunocytochemical evaluation of effusions. We find vimentin to be one of the important components of a basic immunopanel for interpretation of the SCIP.<sup>[2]</sup>

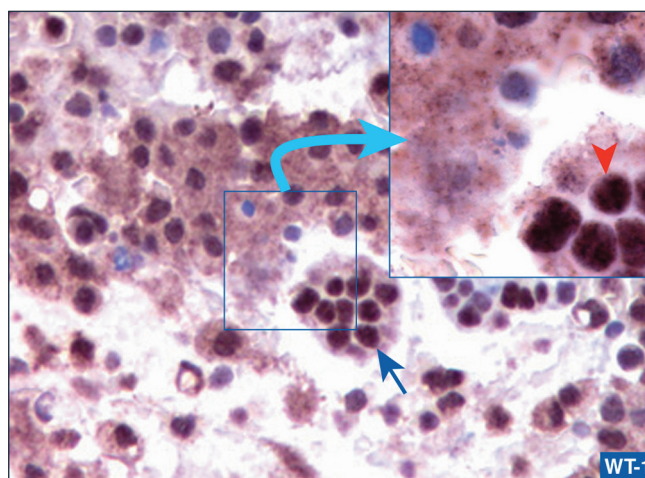
## WT-1 (WILMS' TUMOR GENE PRODUCT)

**Clones/synonyms:** 6F-H2, C-19

WT-1 has a nuclear immunoreactivity pattern (Figure 20). Tissue studies demonstrated WT-1 immunoreactivity in 72% of mesotheliomas, 100% of non-neoplastic pleural mesothelium, and 83% of ovarian adenocarcinomas. Immunoreactivity was observed rarely with all other types of adenocarcinomas.<sup>[50,52,60,87]</sup> Thus, this immunomarker may be more specific in non-peritoneal effusions, especially in males. Studies with effusions are indicated to verify the role of this



**Figure 19:** *Vimentin* immunoreactivity pattern (peritoneal wash). Reactive mesothelial cells (arrow RM) show cytoplasmic immunoreactivity pattern (arrowhead in inset). [Immunostained cell-block section (40X).]



**Figure 20:** *WT-1* immunoreactivity pattern (metastatic colonic adenocarcinoma, peritoneal fluid). Reactive mesothelial cells (arrow RM) show nuclear immunoreactivity (arrowhead in inset) with some cytoplasmic immunostaining. Rare adenocarcinoma cells demonstrating nuclear immunoreactivity for CDX2 were also seen in other section. [Immunostained cell-block section (40X).]

immunomarker. WT-1 immunoreactivity is also observed with desmoplastic small round cell tumors (DSRCT).<sup>[115]</sup>

## LIST OF ABBREVIATIONS (In alphabetic order)

AdCa – Adenocarcinoma  
 AP – Alkaline phosphatase  
 Ca – Carcinoma  
 CK – Cytokeratin  
 DAB – 3,3'-Diaminobenzidine

DSRCT – Desmoplastic small round cell tumors  
 GI – Gastrointestinal  
 HIER – Heat induced epitope retrieval  
 HRP – Horseradish peroxidase  
 LBC – Liquid-based cytology  
 PAP – Papanicolaou  
 RTU – Ready to use  
 SCIP – Subtractive coordinate immunoreactivity pattern  
 m – Monoclonal  
 Min – Minutes  
 p – Polyclonal  
 RCC – Renal cell carcinoma  
 UV – UltraView

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