The Egyptian Journal of Hospital Medicine (July 2023) Vol. 92, Page 5968- 5976

Role of Immunohistochemistry in Pleural Effusion Cytology: Review Article

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ABSTRACT

Background: Immunohistochemistry is crucial for the proper diagnosis of malignant mesothelioma, especially in cases with complex morphology and in biopsy and cytology specimens where it is difficult or impossible to determine the tumor architecture. In the vast majority of instances, the correct identification of tumor lineage is made possible by the application of a tailored panel of mesothelial- and epithelial-specific markers.
Methods: The authors review the most commonly used cytologic preparations, fixatives, and antibodies used in effusion ICC.
Results: Through the utilization of cell block preparations and a panel of antibodies appropriate for the differential diagnosis in question, ICC conditions utilized in surgical pathology can be most closely replicated.
Conclusions: Accurate differentiation between malignant mesothelioma and lung adenocarcinoma in pleural effusion cytology is still a big challenge. Therefore, it is mandatory to search for new diagnostic immunohistochemical markers.
Keywords: Immunohistochemistry, Pleural effusion, Cytology.

INTRODUCTION

A diagnostic problem in and of itself can be cytomorphologic distinction of pleural cytology. When the initial locations of tumor cells are unknown or neoplastic cells show only mild atypia, the challenge is made more difficult. It is frequently used to improve accurate cytological diagnosis of bodily fluids. Many antibodies have been utilized to distinguish reactive mesothelial cells from metastatic cancer. However, the focus of clinical research continues to be finding a biomarker with high sensitivity and specificity [(1)].

Like histologic specimens, using immunocytochemical and molecular methods on cell blocks or smears significantly increases diagnostic accuracy [(2, 3)]. It's interesting to note that not all mesotheliomas readily discharge cancerous cells, for example, sarcomatous mesotheliomas are rarely found using effusion cytology [(4)]. Although epithelioid mesothelioma has been distinguished from secondary carcinoma and other malignant tumors that have spread to serosal membranes using "positive" and "negative" immune-histochemical markers, none of these markers requires panels of "positive" antibodies and indicators with negative predictive value for the diagnosis of mesothelioma due to its 100% sensitivity and specificity [(5)].

Overlapping cytologic features of adenocarcinoma, reactive mesothelial cells, and malignant mesothelioma have long been a diagnostic challenge to cytopathologists. Immunocytochemistry assists in reducing false-negative results of effusion cytology that is reported in over 50% of cases in routine cytology. Such diagnosis errors are usually caused by mistaking reactive mesothelial cells for cancer cells. False-positive diagnoses are less prevalent and frequently result from misinterpreting reactive mesothelial cells as cancerous cells [(6)].

A morphologic examination's sensitivity for detecting metastatic cancer in depending on the cytopathologist's skill and the quality of the preparations, effusions can range from 40% to 80%. It was shown that the specificity increases and the sensitivity increases from 84 to 94% and from 92 to 100% when cytomorphology and immunocytochemistry are coupled [(7)].

Calretinin, D2-40, and CK5/6 are three immunohistochemical markers that have been shown to distinguish malignant mesothelioma from other types of cancer. Ber-EP4, CEA, and TTF1 were markers for adenocarcinomas, and Ber-EP4, CK5/6, and CEA were indicators for lung squamous carcinoma [(8)], the usage of a panel of antibodies ensures the accuracy and increases the sensitivity and specificity of the diagnostic tool [(9)].

The indicators that can currently be used to distinguish between tables (1) and (2) cover epithelioid pleural mesotheliomas from lung adenocarcinomas as well as epithelioid mesotheliomas and squamous cell carcinomas. At least two mesothelial and two carcinoma markers should be used, according to the International Mesothelioma Interest Group in each panel because none of these markers is 100% specific [(10)].

Received:5/2/2023
Accepted:5/4/2023

Table (1): Immunohistochemical markers used in the differential diagnosis between epithelioid pleural mesothelioma and lung adenocarcinoma [(10)]

MARKER	CURRENT VALUE/COMMENTS
Epithelioid mesothelioma (positive mesothelioma markers)	
◆ Calretinin	◆ Very useful. It can be demonstrated in nearly all epithelioid mesotheliomas when antibodies to human recombinant calretinin are used. The staining is often strong and diffuse, and both nuclear and cytoplasmic. Five percent to 10% of lung adenocarcinomas are positive, but the staining is usually focal.
◆ Cytokeratin 5 or 5/6	◆ Very useful. It is expressed in 75% to 100% of the mesotheliomas. Approximately 2% to 20% of lung adenocarcinomas can be focally positive.
◆ WT-1	◆ Very useful. Approximately 70% to 95% of the mesotheliomas show nuclear positivity. Lung adenocarcinomas are negative.
◆ D2-40 (podoplanin)	◆ Very useful. Approximately 90% to 100% of mesotheliomas show positivity along the cell membranes. Up to 15% of lung adenocarcinomas are focally positive.
Lung adenocarcinoma (positive carcinoma markers)	
◆ MOC-31	◆ Very useful. Approximately 95% to 100% of lung adenocarcinomas are positive. Two percent to 10% of mesotheliomas show focal staining.
◆ BG8 (LewisY)	◆ Very useful. Approximately 90% to 100% of lung adenocarcinomas are positive. Three percent to 7% of mesotheliomas show focal reactivity.
◆ CEA (monoclonal)	◆ Very useful. Approximately 80% to 100% of lung adenocarcinomas are positive. Fewer than 5% of mesotheliomas are focally positive.
◆ B72.3	◆ Very useful. Seventy-five percent to 85% of lung adenocarcinomas are positive. Very few mesotheliomas are positive.
◆ Ber-EP4	◆ Very useful. Ninety-five percent to 100% of lung adenocarcinomas are strongly positive. Up to 20% of mesotheliomas are focally positive.
◆ TTF-1	◆ Very useful. Seventy-five percent to 85% of lung adenocarcinomas show nuclear positivity. It is not expressed in mesotheliomas.
◆ Napsin A	◆ Very useful. Eighty percent to 90% of lung adenocarcinomas show cytoplasmic staining. It is not expressed in mesotheliomas.

Abbreviations: BG8, blood group 8; CEA, carcinoembryonic antigen; TTF-1, thyroid transcription tactor-1; WT-1,Wilms tumor 1.

Table (2): Immunohistochemical markers used in the differential diagnosis between epithelioid pleural mesothelioma and squamous carcinoma of the lung [10].

Marker	Current Value/Comments
Epithelioid mesothelioma (positive mesothelioma markers)	
WT-1	Very useful. Up to 95% of mesotheliomas show nuclear positivity. Lung squamous carcinomas are negative.
Calretinin	Somewhat useful. Virtually all mesotheliomas are positive, often strongly and diffusely, with nuclear and cytoplasmic staining. Approximately 40% of lung squamous carcinomas are positive, but the staining is often focal.
D2-40 (podoplanin)	Not useful. Approximately 80% to 100% of mesotheliomas are positive. Fifty percent of lung squamous carcinomas also stain.
Cytokeratin 5 or 5/6	Not useful. It is expressed in 75% to 100% of mesotheliomas and 100% of lung squamous carcinomas.
Lung squamous carcinoma (positive carcinoma markers)	
p63 or p40	Very useful. One hundred percent of lung squamous carcinomas show strong and diffuse nuclear positivity. Seven percent of mesotheliomas react, often focally.
MOC-31	Very useful. Ninety-seven percent to 100% of lung squamous carcinomas are positive. Two percent to 10% of mesotheliomas show focal staining.
BG8 (LewisY)	Very useful. Eighty percent of lung squamous carcinomas are positive. Three percent to 7% of mesotheliomas show focal staining.
Ber-EP4	Useful. Approximately 85% to 100% of lung squamous carcinomas are positive. Up to 20% of mesotheliomas are focally positive.
Cytokeratin 5 or 5/6	Not useful. One hundred percent of lung squamous carcinomas and 75% to 100% of mesotheliomas are positive.

BG8, blood group 8; WT-1, Wilms tumor 1.

In a meta-analysis, **He *et al.*** [11] discovered that D2-40's combined sensitivity and specificity for MM diagnosis were 0.86 and 0.77, respectively, suggesting a rate of missed diagnoses of 14% and misdiagnoses of 23% that may be insufficient malignant mesothelioma (MM) by itself to diagnose. When interpreting the results of D2-40 immunostaining, additional markers' results should also be considered [11].

The Ber-EP4 antibody has demonstrated excellent specificity and sensitivity for the diagnosis of metastatic cancer. Although certain examples, there are recorded cases of mesothelium with BER-EP4, although the majority of these patients only displayed little or undetectable staining of the mesothelium. However, in rare instances, cytological and histological specimens may be strongly positive [12].

About 85% of lung adenocarcinoma and thyroid cancer patients express TTF-1 positively. However, a TTF-1 positive result may no longer be able to rule out malignant mesothelioma [8].

So, Novel promising marker combinations also need to be added to the literature to improve diagnostic accuracy.

HEG homolog 1 (HEG 1)

The development of molecular targeted therapy for MM has been hampered by the lack of highly specific markers for the disease. Here, we demonstrate that HEG1, a newly discovered mucin-like membrane protein, is a highly specific MM marker [13].

HEG homolog 1(HEG1) It has been proposed that a mesothelioma-related antigen was first identified in 2003 where Marble and colleagues reported that the endothelial cell signaling pathway is regulated by the heart of glass gene, which also controls the concentric expansion of the zebrafish heart with EGF-like domains. HEG1 expression has also been proposed as a possible explanation that could assist mesothelioma cell

survival and growth as well as hepatocellular carcinoma metastasis [14].

Vascular endothelial cells are joined together at junctions made of a variety of proteins. The junctions act as a barrier that regulates the passage of particular molecules, including water, through the vessel wall to manage the growth of the blood vessel. The proteins "Heart of Glass" (HEG1) and Rasip1 are essential for the proper development of the heart and blood arteries. [15].

The membrane protein known as HEG homolog 1 (HEG1) resembles a mucin and has domains that are comparable to those in epidermal growth factor. Through abnormal signaling, including that which takes place during cell adhesion, as well as by providing protection against tumor cell invasion. According to **Tsuji *et al.*** [13], HEG 1 is an antigen connected to mesothelioma [16].

An effective cancer-related antigen is a membrane-anchored protein that resembles mucin and has been heavily modified with glycans. In tumor cells, abnormal processes of carbohydrate chain synthesis result in the production of immature glycans, which are clustered on proteins that resemble mucins. To evaluate serologic tumor markers, certain monoclonal antibodies (mAbs) that recognize atypical glycan clusters are utilized in clinical settings. Furthermore, malignant tumor cells can be precisely identified by combining the identification a protein with mucin-like properties and an erratic glycan attachment. a membrane protein like mucin that is particular to tumors may also be a target for immunotherapy using antibodies or for pharmacological suppression of cell growth. MM may contain a mucin-like membrane protein with unique glycosylation, making it a possible target for molecular-targeting therapy or a correct MM diagnosis [13].

The anti-mesothelioma mAb SKM9-2 can identify the sialylated protein HEG homolog 1 (HEG1). Human HEG1 is a 400 kDa membrane protein that is present on mesothelioma cells. It is heavily O-glycosylated and comprises about 70% of the molecule, however it does not contain tandem repeat sequences. Mesothelioma cell development is associated with the expression of the protein HEG1, which is found on the apical cell surface. Because SKM9-2 can detect mesothelioma more precisely and sensitively than other antibodies against, it would likely be helpful for the accurate detection and diagnosis of malignant existing mesothelioma markers [17].

Even sarcomatous and desmoplastic MM can be found using a monoclonal antibody to sialylate HEG1 and SKM9-2. SKM9-2's sensitivity and specificity to MM were 99% and 92%, respectively. It had no reaction with healthy tissues. The sialylated O-linked glycan was recognized by SKM9-2 in conjunction with the HEG1 peptide leading to this precise distinction. Additionally, we discovered that HEG1 gene silencing dramatically reduced the ability of mesothelioma cells to live and reproduce. This finding implies that HEG1 might make a viable target for drugs that inhibit function. All things considered, our research points to the possibility that sialylated HEG1 could be a useful diagnostic and therapeutic target for MM [13]. HEG1 expression in malignant effusion is furthermore evaluated in this study.

Claudin 4

To keep the fluid balance in the lung, between the airspaces and the fluid-filled tissues in the lung, there needs to be an appropriate barrier. As a barrier to water and solutes, epithelial cells, are essential for maintaining the proper balance of lung fluid [18]. Tight junctions between lung epithelial cells act as a barrier to prevent solutes from freely diffusing into the air spaces. Epithelial barrier function depends on claudins that are transmembrane tight junction proteins. Claudins are controlled by their interactions with one another, which are coordinated with those of cytosolic scaffold proteins and other transmembrane tight junction proteins. There are 14 claudins that are expressed by the alveolar epithelium, whereas claudin-3, claudin-4, and claudin-18 are the most prevalent ones. Each one improves the alveolar barrier function. It has been discovered that claudin-4 plays a protective effect, particularly in preventing lung injury [19]. Claudins are a key component tight intersections. These transmembrane proteins carry out a number of tasks such as recruiting and governing of cell proliferation and differentiation, as well as signaling proteins, at the interfaces between epithelial and endothelial cells. It is well known that tight junction disruption occurs during carcinogenesis. Claudin-4 displays a negative staining pattern in both normal and neoplastic mesothelium and is overexpressed in epithelial neoplasms during neoplastic transformation [20].

Diagnostic challenges of cytology in the diagnosis of pleural effusion

A cytological smear has a 60% diagnosis rate, it will assist with pleural effusion diagnosis as well as determining the stage of cancer and assessing the severity of the condition [21, 22]. Conventional cytological smears, on the other hand, can present diagnostic challenges because they have a lower diagnostic yield, especially with poorly differentiated malignancies [23].

Diagnostic challenges with benign effusion:

High cellularity, many mitotic figures, cytologic atypia, necrosis, and papillary structure group development, reactive mesothelial proliferations resemble mesothelioma (or metastatic cancer). Between benign reactive and malignant mesothelial cell proliferations, there is a significant overlap in atypical features and immunoreactivity, however the wide range of sensitivity (high false-negative rate) is likely caused by sampling rather than assessment. There are some cytologic similarities between reactive and malignant epithelioid mesothelial cells, including scalloped borders around cell clumps, intercellular windows with

lighter, dense cytoplasm margins, and low nuclear to cytoplasmic ratios [24].

Reactive mesothelial cells are referred to as actively dividing mesothelial cells in response to injury or stimuli. Mesothelial cells that react (Figures 1 & 2) reveal a range of modifications that may resemble cancer. These alterations could involve mitotic figures, an expanded nucleolus, and a coarsened chromatin structure. In these situations, cytomorphology should be carefully analyzed, along immunocytochemistry and clinical data are used to resolve the diagnosis conundrum dilemma [25].

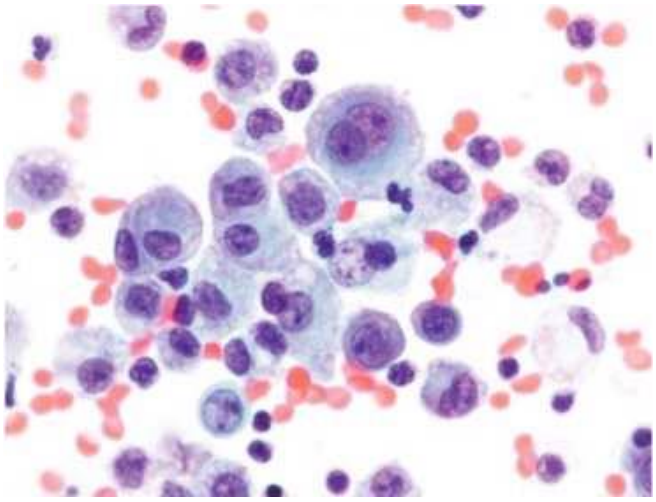

Figure 1: Reactive mesothelial cells. This population of mesothelial cells are relatively enlarged in size and have large, hyperchromatic, irregular nuclei [25].

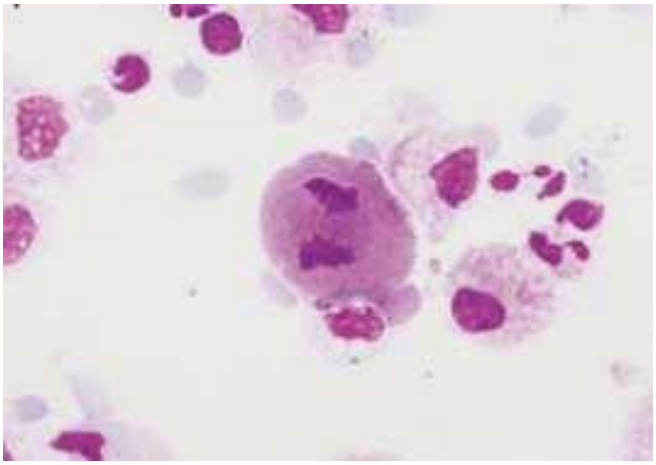

Figure 2: Reactive mesothelial cells show mesothelial cell in mitosis [25].

Separating bland adenocarcinoma cells and macrophages from mesothelial cells are two of the most often encountered problems in serous fluid cytology. The former typically exhibit less extensive microvacuolation in their cytoplasm and more smooth, small, bean-shaped nuclei are present. When a background population of reactive mesothelial cells cannot be identified, the latter can be more challenging. In each of these situations, immunocytochemistry may be required to determine the type of cells present [26].

Mesothelial cells can be recognized by immunocytochemistry because they typically exhibit the immunocytochemical markers CK 5/6, calretinin, and thrombomodulin and also exhibits positive staining in malignant mesothelial cells. But, usually they do not stain with epithelial membrane antigen (EMA), or if they do, they rarely do so, the staining is faint [25].

Diagnostic challenges with malignant mesothelioma in pleural effusion cytology

Cytology is typically used to diagnose the epithelioid type. While, some support the use of cytology in diagnosis, others argue that its utility is restricted because morphological evidence of tissue invasion is required for a reliable diagnosis. This is a little unexpected considering that many other malignant tumors are identified cytologically without recognizing tissue invasion. But, compared to metastatic adenocarcinoma cells, mesothelioma cells are usually plain in fluid samples and are more likely to be overlooked, which adds to the challenge of detection [26].

Often, in serous cavity fluids, large numbers of malignant cells with well-developed cytomorphologic features will be present in MM patients' tissues. However, when the number of lesional cells is modest or the cytomorphology considerably resembles metastatic adenocarcinoma (a frequent scenario), diagnostic difficulties can occur [27]. Even for cytologists with experience, diagnosing mesothelioma in cytological preparations can be difficult since, in some situations, malignant mesothelial cells might resemble reactive mesothelial cells quite closely. This closeness frequently results in a misdiagnosis as negative. To ensure effective treatment, malignant mesothelioma and metastatic malignancy must be differentiated therefore after detecting malignant cells, the next diagnostic hurdle is determining their mesothelial origin. To answer these diagnostic conundrums, we frequently use immunocytochemistry [25].

The cancerous cells in mesotheliomas resemble normal mesothelial cells. It becomes challenging to label cells as malignant based just on morphology if they have achieved a level of differentiation that allows them to be identified as mesothelial. On the other hand, in a suitable clinical scenario, malignant mesothelioma may be diagnosed by the pathologist as a result of atypia in reactive proliferations. The presence of "more and bigger cells in more and bigger clusters" is a crucial indicator in order to identify malignant mesothelioma. Malignancy is indicated by high cellularity and numerous big aggregates, particularly in pleural effusions. Less mesothelial cells and smaller, less complicated clusters can be seen in benign effusions. Malignant proliferations are more likely to have cell-in-cell configurations. Macronucleoli tend to be cancerous [7]. Malignant mesothelial cells may simulate mesothelial cells that are reactive (Table 3). The cells of mesothelioma are in large groups, with moderate nuclear pleomorphism and often varies [28].

Table (3): Comparison of reactive mesothelial cells and mesothelioma [28]

Features	Reactive mesothelial cells	Mesothelioma
Cell cluster	Small loosely adhesive	Large clusters of cells, more than 10–12 cells
Cell size	Small	Relatively large
Nuclear pleomorphism	Mild	Moderate
Nucleoli	Small	Large
EMA	Weak positive	Strong positive
Desmin	Positive	Negative
Glucose-transport protein 1 (GLUT-1)	Negative	Positive
BAP1 expression	Present	Absent in two-thirds cases of mesothelioma

Another challenge for the cytologist is to differentiate adenocarcinoma from mesothelioma. Cells from both mesothelioma and adenocarcinoma may show almost similar morphology. In this situation, the cytologist should depend on (1) clinical history; (2) radiological features; and (3) ancillary tests, such as ICC [28] (Table 4)**.**

Table (4): Distinguishing features between malignant mesothelioma and metastatic adenocarcinoma [28]

Distinguishing points	Malignant mesothelioma	Metastatic adenocarcinoma
Two cell population	Absent	Present
Cell clusters	Spherical clusters with knobby edges	Hollow clusters
Cell-in-cell (cell cannibalism)	Frequent	Less common
Window-like gaps	Present	Absent
Cytoplasmic peripheral halo	Present	Absent
Nucleocytoplasmic ratio	Low	High
Nuclear pleomorphism	Mild	Moderate
Nuclear position	Central	Central to eccentric
Multinucleated giant cells	More frequent	Less frequent
Cytochemistry: • Mucin stain (mucicarmine) • Alcian blue	 • Negative • Positive and diastase sensitive	 • Positive • Positive and diastase resistant
Electron microscopy: Intermediate filaments Microvilli	 Abundant Long	 Scanty Short
Immunocytochemistry: • Calretinin • WT1 • D2-40 • CEA • BER-EP4 • Leu-M1 • B72.3 • Vimentin	 • Positive • Positive • Positive • Negative • Negative • Negative • Negative • Positive	 • Negative • Negative • Negative • Positive • Positive • Positive • Positive • Negative

Diagnostic challenges with metastatic lung carcinoma in pleural cytology:

Adenocarcinoma challenges

The spaces between neighboring mesothelial cells that resemble slits are known as "windows" and appear as mesothelial cells combine together. They are an image of the long, thin microvilli that cover their surface. The presence of "windows" in a collection of cells provides information about their mesothelial ancestry. It should be emphasized, though, that adenocarcinoma cells can also be seen to have "windows" between them. According to two independent investigations, "windows" were seen in 13 and 44% of adenocarcinoma cases, and mucus secretion was typically the cause of the slit-like crevices between neoplastic cells in these cases [29, 30] (Figure 3).

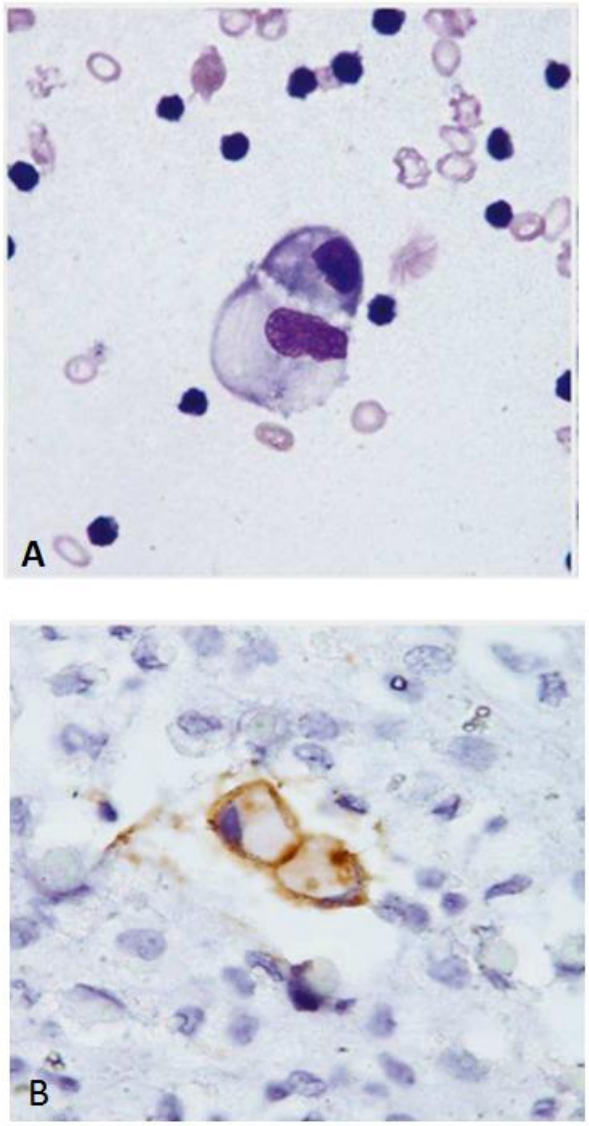

Figure (3): (A)Slit-like space between two adenocarcinoma cells looking like “windows” of the mesothelial cells, MGG. (B) Ber-EP4 positivity in these carcinoma cells [7].

The curves of mesothelial groupings generally resemble flowers (Figure 4). In contrast, adenocarcinoma cells form groups with common borders, such as cell balls and papillae. Knobby-contoured cell clusters are a feature of mesothelial cells both seen in reactive proliferation and in malignant mesotheliomas. However, not infrequently (36.9%), they may also be present in adenocarcinomas (Figure 6). On the other hand, in some cases of mesothelial hyperplasia, papillary structures may develop, creating a pitfall in the differential diagnosis (Figure 5) [7].

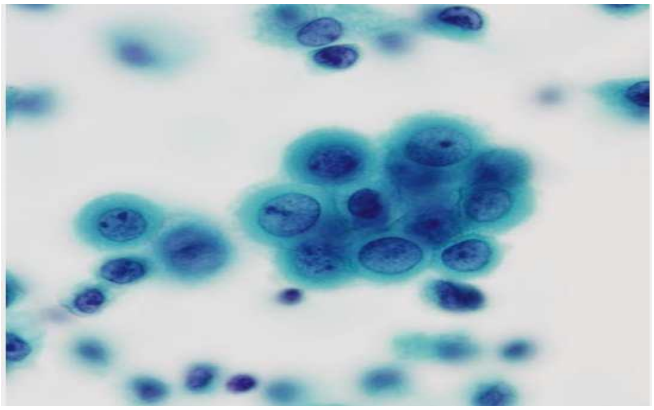

Figure (4): Knobby contours of mesothelial clusters; PAP [7].

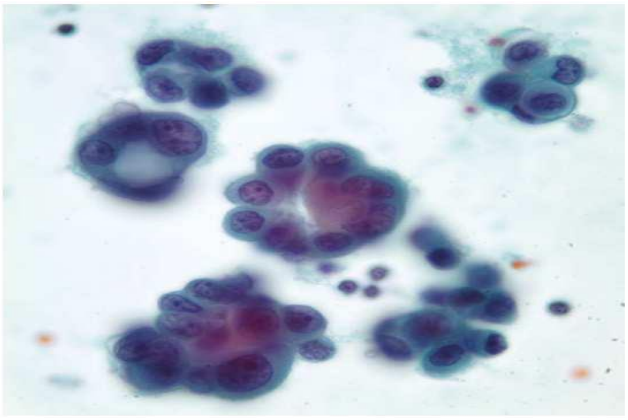

Figure (5): Reactive mesothelial cells forming papillary structures; PAP [7].

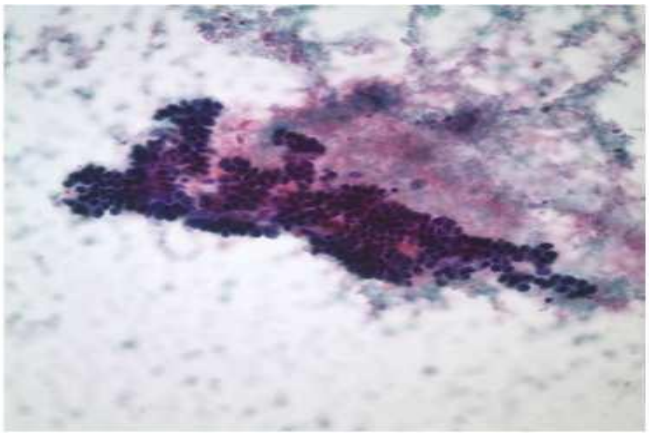

Figure (6): Adenocarcinoma displaying groups with knobby contours; PAP [7].

- **Competing interests: None**
- **Funding: No fund**

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