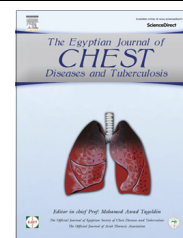




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## ORIGINAL ARTICLE

# Combined serum and immunohistochemical differentiation between reactive, and malignant mesothelial proliferations



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### KEYWORDS

Reactive mesothelial proliferation;  
 Atypical mesothelial proliferation;  
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 Desmin;  
 Epithelial membrane antigen (EMA);  
 Serum mesothelin related peptide (SMRP)

**Abstract** *Background:* Malignant mesothelioma (MM) carries a poor prognosis and response rates to palliative chemotherapy remain low. The diagnosis of malignant mesothelioma is frequently difficult, the most common differential diagnosis being reactive pleural conditions and metastatic adenocarcinoma. Several studies have used immunohistochemical markers to distinguish between reactive and neoplastic mesothelial cells. Soluble mesothelin levels in serum have recently been shown to be highly specific and moderately sensitive for mesothelioma. A combined detection of serum levels of mesothelin and immunohistochemical expression of desmin and EMA are used in order to differentiate between reactive mesothelial proliferations, and malignant mesothelioma of epithelioid type.

*Patients and methods:* This prospective study includes 17 cases of reactive mesothelial proliferations, 6 cases of atypical mesothelial proliferations and 13 cases of MM. Cases were collected from the Chest Department, Faculty of Medicine, Benha University and International Medical Center (IMC), in the period 2012–2014. Desmin and epithelial membrane antigen (EMA) immunohistochemical staining were performed in all cases and the pattern of expression was analyzed. Soluble mesothelin related peptide (SMRP) was estimated for all cases.

*Results:* Desmin expression was positive in 88.2%, 0%, and 7.7% of reactive mesothelial proliferations, atypical mesothelial proliferations and MM respectively. EMA was positive in 5.9% of

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reactive mesothelial proliferation, 100% of atypical mesothelial proliferations and 92.3% of MM cases ( $P < 0.01$ ). The calculated mean SMRP was 6.6 nM. SMRP levels were higher than the calculated mean value in 17.6% of studied reactive mesothelial lesions, 66.7% and 76.9% of atypical mesothelial proliferations and MM respectively, which was statistically highly significant correlation ( $P < 0.01$ ).

**Conclusion:** Combined estimation of SMRP level and immunohistochemical detection of both EMA and desmin could be a useful tool for differentiation between reactive mesothelial proliferation and malignant mesothelioma.

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## Introduction

Malignant mesothelioma (MM) is an aggressive asbestos-related cancer of serosal surfaces such as the pleura, peritoneum and rarely the pericardium. The cell of origin is a sub-mesothelial mesenchymal stem cell. It is causally linked to asbestos exposure [1]. According to the Egyptian National Cancer Institute (NCI), MM constituted 13.12% of recorded respiratory system tumors and 0.84% of total recorded malignancy. The ratio between malignant lung tumors and pleural mesothelioma was 1.8:1. Pleural mesothelioma showed a wide age range starting from the 3rd to the 8th decade. However the majority of the cases were between 30 and 70 years. Epithelioid mesothelioma constituted 45.13% of all recorded mesotheliomas [2,3]. Diagnosis of MM is challenging as symptoms and early radiographic signs are often non-specific and their significance can be masked by multiple co-morbidities of this normally older patient. Malignant pleural mesothelioma has a median survival of seven to ten months and a clinical pattern that usually involves substantial pain and dyspnea. It presents at a clinically advanced stage in most patients so there is a need for new methods of early detection [4].

Mesothelial cells frequently show florid reactive changes in response to many benign conditions such as pulmonary infarction, systemic disease (i.e., collagen-vascular diseases), cirrhosis, radiation, underlying neoplasm, chronic inflammation, foreign substance, and infection. The distinction between benign reactive mesothelial proliferations and malignant mesothelioma (MM) may be very difficult based only on histologic and morphologic findings. Because of the difficulty in distinguishing reactive conditions from MM even in tissue specimens, such as small pleural biopsies, several studies have used immunohistochemical markers to distinguish between reactive and neoplastic mesothelial cells [5].

The intermediate filament protein desmin is a known marker for smooth and skeletal muscle differentiation. Several studies have reported positive staining of benign mesothelial cells (reactive mesothelial proliferation) in serous fluid and tissue sections for desmin. The exact etiology for expression of desmin in mesothelial cells is not known; however, the multi-potential role of mesothelial cells with possible muscle differentiation and coexpression of desmin has been proposed by some studies [6,7].

Epithelial membrane antigen (EMA) is one of several glycoproteins found in human milk fat globule membranes. The glycoprotein identified with EMA is known to be one of a series of glycoproteins or mucins and is designated MUC1 [5]. It is a high molecular weight transmembrane glycoprotein

expressed in cancer cells that suppresses cellular aggregation and cell-matrix adhesion and promotes invasion of extracellular matrix by malignant cells. Moreover, it inhibits T-cell mediated cytotoxicity through either induction of apoptosis in activated T cells or inhibition of cytotoxic lymphocyte-target cell interactions. MUC1 is also a ligand for ICAM-1 immunoglobulin which is expressed on endothelial cells. This allows intravascular tumor cells to adhere to and invade through the endothelial barrier; facilitating metastatic spread [7,8].

Mesothelin is a 40 kDa membrane-localized protein that along with the 31 kDa megakaryocyte potentiation factor (MPF) are cleavage products of a 69 kDa precursor protein encoded by MSLN gene on chromosome 16. In tissue culture, Mesothelin is proposed to play a role in cell adhesion as it binds to the cell adhesion molecule Ca125 (Muc16) and forced over-expression of MSLN leads to increased adhesion to a plastic substrate [9,10]. Also in tissue culture, mesothelin promotes cell proliferation, invasion and apoptosis resistance. Mesothelin may therefore be involved in cancer metastasis and its role as a potential therapeutic target is being actively pursued. It is predominantly expressed in epithelioid subtype mesotheliomas, with little/no expression in sarcomatoid subtypes. MPF and mesothelin isoforms 1 and 3 can be detected as soluble proteins in plasma or serum, which may be detected using a validated commercial dual antibody ELISA platform [11,12].

The small amount of mesothelin shed into the serum could make it a valuable diagnostic tool in cancers that express mesothelin. It has been shown to potentially differentiate between mesothelioma and other conditions, both benign and malignant, and also potentially correlates with response to therapy [1,13].

A study by Marchevsky [14] has demonstrated that the use of many markers does not provide higher diagnostic accuracy than the use of selected single antibodies or various combinations of only 2 markers. In this work a combined detection of serum levels of mesothelin and immunohistochemical expression of EMA and desmin are used in order to differentiate between reactive mesothelial proliferations and malignant mesothelioma of epithelioid type.

## Patients and methods

This prospective study included 17 cases of non-neoplastic reactive mesothelial proliferations, 6 cases of atypical mesothelial proliferations and selected 13 cases of malignant mesothelioma; epithelioid type. Thoracoscopic biopsies were collected

from the Chest Department, Benha Faculty of Medicine – Benha University and the International Medical Center (IMC) in the period (June 2012–June 2014). Paraffin-embedded tissue sections were prepared from obtained biopsies. Hematoxylin and Eosin sections were reviewed by two pathologists to confirm diagnosis.

#### *Immunohistochemical staining*

Tissue sections were mounted on positively-charged slides, steps of staining followed the standard ABC (avidin–biotin complex) procedure using the Ultra Vision Detection System (Anti-polyvalent, HRP/DAB, ready-to-use, Lab Vision corporation). Antigen retrieval was done with microwave treatment in 10 mM citrate buffer (Neo-Markers, Cat. # AP-9003), pH 6.0. Sections were incubated with rabbit monoclonal antibody desmin (Lab Vision, Thermo scientific, USA, Cat. # RB-9014-P0, 1:200 dilution) and with mouse monoclonal antibody EMA (Lab Vision, Thermo scientific, USA, Cat. # MS-741-P0, 1:200 dilution). The incubation and pretreatment time were 30 min at room temperature for both antibodies. The freshly prepared DAB-substrate-chromogen solution was applied.

#### *Immunostaining interpretation*

Sections were evaluated under a light microscope and desmin positivity was considered as brown cytoplasmic staining. The results for desmin immunohistochemical stains were recorded as negative when no immunoreactivity was seen, focal/weak if < 20% of cells were positive or showed only blush positivity, and positive if strong positivity was seen in  $\geq 20\%$  of cells [6].

Only membranous staining was regarded as positive for EMA. The results for EMA immunohistochemical staining were recorded as negative (no staining), focal/weak if there were a few (< 20%) scattered cells that showed a membranous staining pattern or if there was only blush cytoplasmic staining but no membranous staining, and positive if there were  $\geq 20\%$  of mesothelial cells that showed strong membranous accentuation and cytoplasmic staining [6].

Normal muscle tissue was taken as a control for positive desmin expression and non-neoplastic breast tissue served as a positive control for EMA. Negative controls were performed by replacing the primary antibody with normal rabbit nonimmune IgG [6,8].

#### **Mesothelin assay**

The serum mesothelin assay was performed in a single laboratory. Serum samples were prospectively collected alongside clinical data. Levels of serum mesothelin were assayed with a commercial ELISA kit (Mesomark™ Fujirebio Diagnostics, Malvern PA) according to the manufacturer's instructions. The MESOMARK assay was performed according to the manufacturer's instructions. Briefly, patient serum samples were diluted 1:101 using the assay diluent provided and 100 L of the diluted samples were added in duplicates to a 96-well plate precoated with the 4H3 antibody. The samples were incubated on a plate-shaker for 60 min followed by a 5 rinse with wash buffer. The OV569-HRP conjugate was next added to the sample wells and the microwell plate incubated for a further 60 min on a plate-shaker. After a wash step,

100 L of substrate was added to the reaction wells for 15 min before adding 100 L of stop solution. The absorbance at 450 nm was used to quantify the soluble mesothelin-related protein (SMRP) levels by comparison to a six-point calibration curve. The MESOMARK values are expressed as nM (nanomolar). Results were expressed in nanomoles per liter (nmol/L). All analyses were performed in a batch, blinded to clinical outcomes [15].

#### *Statistical analysis*

Data analysis was performed with the statistical package for social sciences (version 16.0.1; SPSS Inc., Chicago, Illinois, USA). Descriptive analysis of the variables and statistical significance of the tests were expressed in *P*-value. *P* value less than 0.05 (< 0.05) was considered significant and < 0.01 was highly significant.

#### **Results**

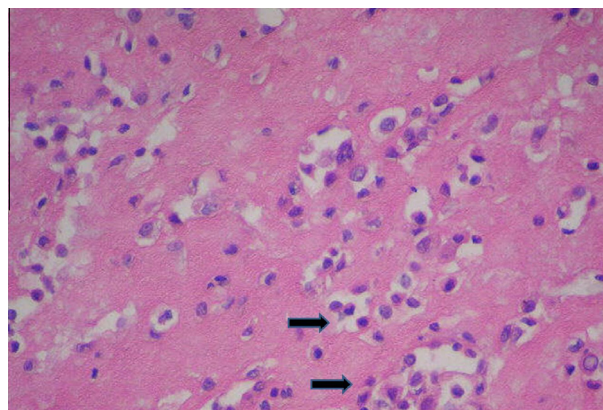
*Histologically*, reactive mesothelial proliferations were defined by relatively bland monomorphic cuboidal cells. These cells have normal nuclear to cytoplasmic ratio, regular chromatin pattern, with or without distinct nucleoli. Associated inflammatory reaction is sometimes present (Fig. 1).

The cells of atypical mesothelial proliferations are cuboidal to elongate showing varying degrees of cytologic atypia with enlarged nuclei, and often have prominent nucleoli with significant cell-to-cell variation. In some cases atypical cells form a single layer along the pleural surface, in other cases they tend to form linear arrays between layers of fibrin (Fig. 2).

Malignant mesothelioma cells show conspicuous malignant cytologic features (severe pleomorphism, abnormal mitoses). The tumor cells are arranged in solid nests or pseudoacini and are surrounded by dense fibrous tissue reaction. All cases of MM included in this study were of epithelioid type with invasion of underlying tissue (Fig. 3).

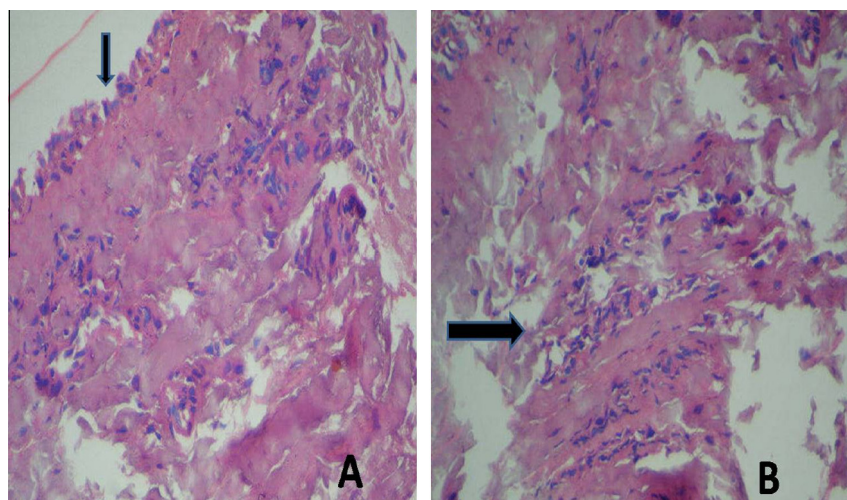
#### *Immunohistochemical results*

Fifteen cases (88.2%) of reactive mesothelial proliferations were positive to desmin expression in the form of diffuse

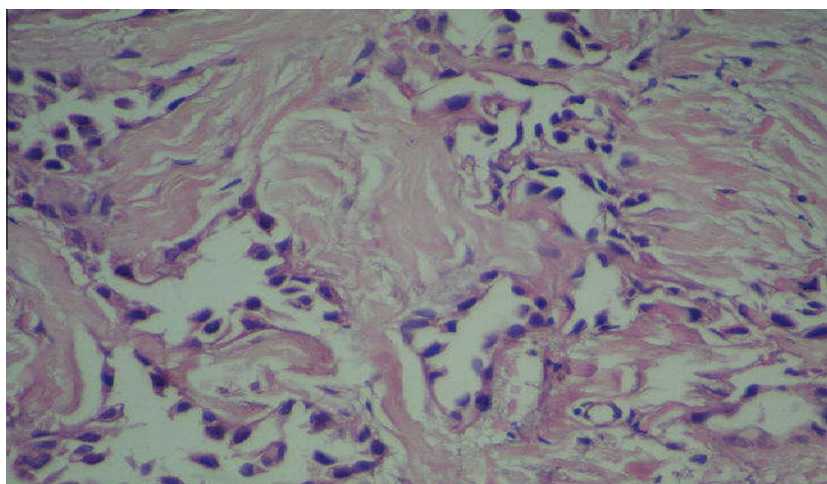


**Figure 1** Reactive mesothelial proliferation showing relatively bland monomorphic cuboidal cells with normal nuclear to cytoplasmic ratio (arrows). Few inflammatory cells are seen (H&E  $\times 400$ ).





**Figure 2** Atypical mesothelial proliferation showing cuboidal to elongate showing varying degrees of cytologic atypia. (A) Atypical mesothelial cells arranged on the pleural surface (thin arrow). (B) Atypical mesothelial cells tend to form linear arrays between layers of fibrin (thick arrow) (H&E  $\times 400$ ).



**Figure 3** Malignant mesothelioma epithelioid type, showing malignant mesothelial cells arranged in pseudoacini and surrounded by dense fibrous tissue reaction. The cells show conspicuous malignant cytologic features (severe pleomorphism, marked hyperchromasia and abnormal mitoses) (H&E  $\times 400$ ).

cytoplasmic staining (Fig. 4A). One case (16.7%) of atypical mesothelial proliferations showed weak desmin expression and one case (7.7%) of MM was positive to desmin immunostaining (Figs. 5A and 6A). There was a statistically highly significant inverse correlation ( $P < 0.01$ ) between desmin expression and type of lesion studied (Table 1).

Regarding EMA expression, one case (5.9%) of reactive mesothelial proliferations was EMA positive (Fig. 4B). All cases (100%) of atypical mesothelial proliferation and 12 (92.3%) cases of MM showed strong membranous accentuation, with some cytoplasmic staining (Figs. 5B and 6B). There was a statistically highly significant correlation ( $P < 0.01$ ) between EMA expression and type of lesion studied (Table 1).

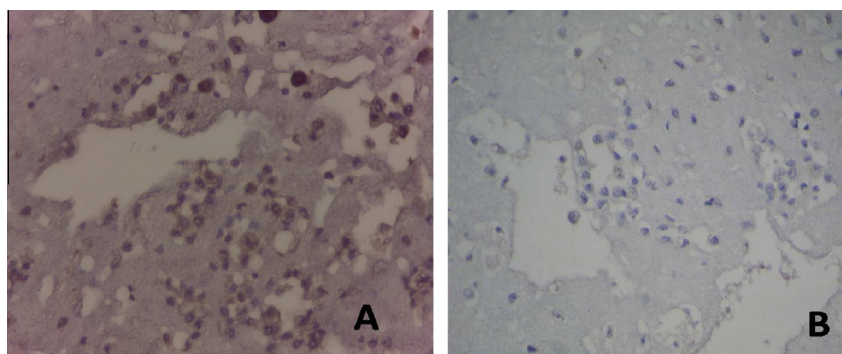
Cases were analyzed for combined immunoprofile of desmin and EMA:

Combination of Desmin +ve/EMA -ve or Desmin -ve/EMA +ve could be used for differentiation between reactive mesothelial proliferations and MM ( $P < 0.01$ ), however, atypical and malignant proliferations had the same immunoprofile and could not be differentiated from each other (Table 2).

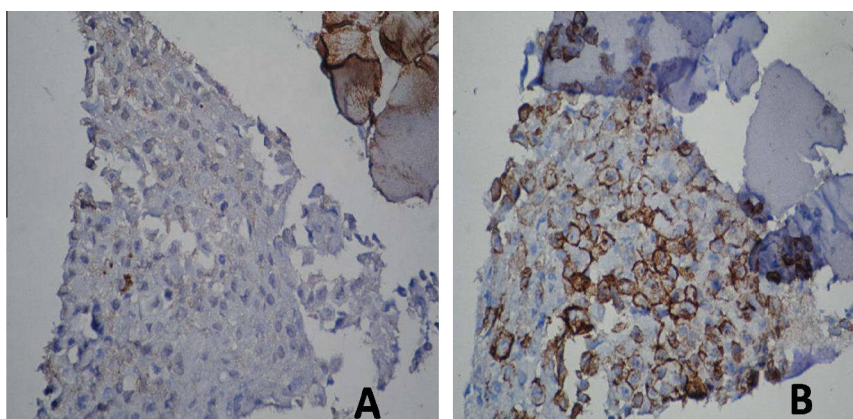
#### Serum mesothelin results

In the 36 patients tested for serum mesothelin the mean level was 6.6 nM (range 0.3–102.5 nM). The value of 102.5 nM appeared to be an outlier. The mean value was chosen a priori and used for subsequent analyses.

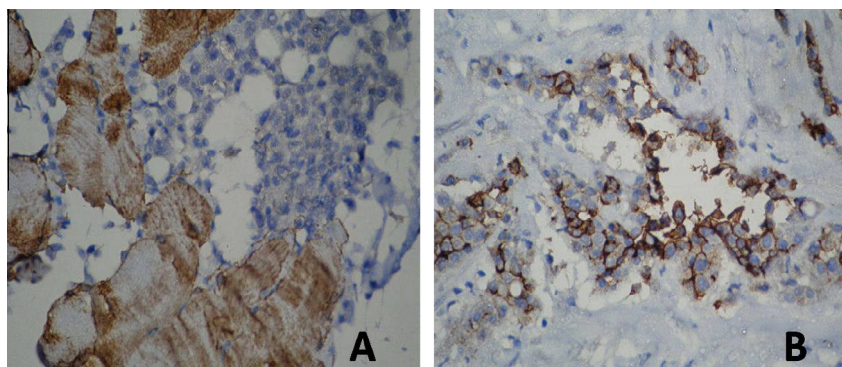
The serum levels of mesothelin related protein were  $\geq 6.6$  nM in 3 cases (17.6%) of reactive mesothelial proliferation, 4 cases (66.7%) of atypical mesothelial proliferation and



**Figure 4** Reactive mesothelial cells showing (A) +ve cytoplasmic expression of desmin. (B) –ve EMA expression (IHC, DAB × 400).



**Figure 5** Atypical mesothelial proliferation showing (A) negative desmin expression (B). Positive EMA expression in the form of strong membranous staining (IHC, DAB × 400).



**Figure 6** Malignant mesothelioma showing (A) –ve cytoplasmic desmin expression. Positive staining was detected in neighboring muscle fibers (B). Positive EMA in the form of strong membranous expression (IHC, DAB × 400).

10 cases (76.9%) of mesothelioma. There was a statistically highly significant correlation ( $P < 0.01$ ) between SMRP level and type of studied cases (Table 3).

**Discussion**

Diagnosis of malignancy on pleural biopsy may be problematic. Reactive pleural processes may be associated with pleural effusion and thickening of the pleura and in some cases raise the clinical possibility of malignancy, whereas some MMs are cytologically bland or even indistinguishable from benign

mesothelial cells or may be sampled in minimally invasive areas that hide their malignant nature. It is of considerable importance to patients to know that they have a benign pleural process rather than MM [14,16]. Because cytologic atypia is not a reliable factor [17], this work investigated a role of immunohistochemical stains to make this distinction.

The current study found that diffuse cytoplasmic staining of desmin was detected in 88.2% of reactive mesothelial proliferations and 7.7% of MM cases. This was a statistically highly significant inverse correlation ( $P < 0.01$ ). Parallel to such results, Hasteh et al., [7] reported that desmin was positive in



**Table 1** Expression of desmin and EMA among studied cases.

	Total	Desmin expression			<i>P</i> value	EMA expression			<i>P</i> value
		Negative	Weak	Positive		Negative	Weak	Positive	
Reactive mesothelial proliferation	17	0	2 (11.8%)	15 (88.2%)	<i>P</i> < 0.01	12 (70.6%)	4 (23.5%)	1 (5.9%)	<i>P</i> < 0.01
Atypical mesothelial proliferation	6	5 (83.3%)	1 (16.7%)	0	<i>P</i> < 0.01	0	0	6 (100%)	<i>P</i> < 0.01
Mesothelioma	13	10 (76.9%)	2 (15.4%)	1 (7.7%)	<i>P</i> < 0.01	0	1 (7.7%)	12 (92.3%)	<i>P</i> < 0.01

**Table 2** Combined immunoprofile of desmin and EMA in relation to type of examined cases.

	Total	Reactive mesothelial proliferation	Atypical mesothelial proliferation	Mesothelioma	<i>P</i> value
Desmin +ve/EMA -ve	12	12 (100%)	0	0	<i>P</i> < 0.01
Desmin +ve/EMA +ve	9	5 (55.6%)	1 (11.1%)	3 (33.3%)	<i>P</i> > 0.05
Desmin -ve/EMA -ve	0	0	0	0	
Desmin -ve/EMA +ve	15	0	5 (33.3%)	10 (66.7%)	<i>P</i> < 0.01

-ve: negative, +ve: positive.

**Table 3** Random soluble mesothelin-related protein levels.

	Total	SMRP level		<i>P</i> value
		<6.6 nM	≥6.6 nM	
Reactive mesothelial proliferation	17	14 (82.4%)	3 (17.6%)	<i>P</i> < 0.01
Atypical mesothelial proliferation	6	2 (33.3%)	4 (66.7%)	<i>P</i> < 0.01
Mesothelioma	13	3 (23.1%)	10 (76.9%)	<i>P</i> < 0.01

SMRP: Soluble mesothelin-related protein.

84% of cases of reactive mesothelial proliferation and in 6% of MM cases (*P* < 0.01). Also Afify et al., [18] found that strong cytoplasmic staining for desmin is observed in 92% of cases of reactive mesothelial cells. In addition, Panjkoviü et al., [19] found that 100% of pleural MM is negative for desmin.

As desmin is considered a marker for smooth and skeletal muscle differentiation, this work suggests loss of muscle differentiation in MM cells. Desmin alone is not completely a reliable marker to differentiate between a reactive and a malignant process in this study, because 7.7% of our MM cases were positive, with an additional 15.4% focally positive.

It is possible that the focal desmin staining in the mesothelioma cases represented a residual population of non-neoplastic mesothelial cells [7].

In the current study, EMA expression was detected in 5.9% of reactive mesothelial proliferations, while all cases (100%) of atypical mesothelial proliferation and 92.3% of cases of MM were EMA positive with strong membranous accentuation, and some cytoplasmic staining. This was statistically highly significant (*P* < 0.01). These results were in agreement with results reported by Haste et al., [7] who found that EMA was positive in 9% of benign mesothelial proliferation and 100% of malignant mesothelioma cases (*P* < .001). Previous studies have shown that strong staining for EMA is helpful in excluding reactive mesothelial cells, although focal and weak positivity has been reported [20].

Shen, et al., [21] reported that EMA is most highly expressed in epithelioid mesotheliomas and rarely in the sarcomatoid subtype. Other reports, however, have shown that reactive mesothelium can be positive for EMA in up to 70% of cases [22]. Different antibody clones may account for these conflicting results. Shen, et al., [21] also have confirmed that EMA is a specific marker of malignancy for mesothelioma when staining is strong and diffuse. Minato et al., [23] reported that EMA is a positive marker for MM which showed sensitivity of 79%, and specificity of 88%.

This study suggested that desmin and EMA not only serve as markers for mesothelial cells in the appropriate setting, but also their combination can aid in distinction between reactive and malignant mesothelial cells. These results are matching with results of Attanoos et al., [20] who concluded that desmin and EMA appear to be the most useful markers in distinguishing benign from malignant mesothelial proliferations. Desmin appears to be preferentially expressed in reactive mesothelium and EMA appears to be preferentially expressed in neoplastic mesothelium. The complementary use of both markers is advocated in ascertaining the nature of mesothelial proliferations.

Conversely, Salman et al., [24] found primary malignant peritoneal mesothelioma with an unusual immunohistochemical profile-desmin positive, EMA negative. In the study of Minato et al., [23], they reported that in MM, the proportion of positive tumor cells ranged from 5% to 100% (59%) for EMA, and 5% for desmin. In reactive mesothelial lesions, the proportion of positive mesothelial cells ranged from 5% to 30% (mean, 12%) for desmin, 10% to 35% (19%) for EMA.

These conflicting results might be attributed to differences in patient populations, specimen types, scoring systems, and anti bodies and antigen retrieval methods. The histologic subtypes of MM included in each study may also affect the results.

In this study, SMRP levels were higher than the calculated mean value (6.6 nM) in 17.6%, 66.7% and 76.9% of reactive mesothelial proliferations, atypical mesothelial proliferations and MM respectively, which was statistically highly significant correlation (*P* < 0.01). Parallel to such results Craeney et al.,

[13] reported that significantly higher levels of mesothelin were found in effusions of patients with mesothelioma; with a specificity of 98%, the assay had a sensitivity of 67% comparing patients with mesothelioma and those with effusions of non-neoplastic origin. Robinson et al., [25] reported in his study on mesothelin family of proteins that patients with malignant mesothelioma had a higher level of mesothelin related peptide than the healthy control. Also Hollevoet et al., [26] reported that in patients suspected of having mesothelioma, a positive blood test for mesothelin at a high-specificity threshold is a strong incentive to urge further diagnostic steps. Similarly Hassan et al., [27] found that elevated serum mesothelin levels were noted in 40 of 56 (71%) patients with mesothelioma. In their meta-analysis study, Luo et al., [28] found that the estimates for SMRPs in the diagnosis of malignant mesothelioma in the studies included were sensitivity of 0.64 (95% confidence interval 0.61–0.68), specificity of 0.89 (0.88–0.90).

*In conclusion* combined estimation of SMRP level and immunohistochemical detection of both EMA and desmin could be a useful tool for differentiation between reactive mesothelial proliferations and malignant mesothelioma of epithelioid type. However atypical proliferations could not be differentiated from MM in this work.

#### Conflict of interest

No conflict of interest.

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