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IMMUNOHISTOCHEMICAL STUDY OF DESMIN, EPITHELIAL MEMBRANE ANTIGEN AND CALRETININ IN PLEURAL FLUID CELL BLOCK PREPARATIONS

Pathology

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ABSTRACT

Background: Distinction between reactive mesothelial cells, malignant mesothelioma and carcinoma is challenging in both biopsy and cytologic material. This study was conducted to differentiate benign/ reactive mesothelial proliferation from malignant mesothelial proliferations and metastatic adenocarcinoma by using immunohistochemical (IHC) markers Desmin (DES), Epithelial membrane antigen (EMA) and Calretinin (CAL) in pleural fluid cell block (CB) preparations. **Methods :** A two year descriptive study (Oct.2016- Sept.2018). 46 pleural fluids samples sent to the Dept. of Pathology, RIMS for routine examination and histopathological examination by CB preparation were studied using IHC markers EMA,DES and CAL following H & E stain. **Results:** Out of 46 cases, 9(19.6%) cases were diagnosed as benign, 23(50.0%) as reactive and 14(30.4%) as adenocarcinoma on H & E section by CB preparations within an age range 34 to 80 years (Mean±SE, 60.32±12.13). Following IHC staining with EMA, DES & CAL, 11(23.9%) cases were confirmed as benign, 17(37.0%) as reactive, 16(34.8%) as adenocarcinoma and 2(4.3%) cases as malignant mesothelioma. **Conclusions:** This study showed that EMA, DES and CAL helpful in confirming benign or reactive mesothelial and malignant mesothelial with epithelial cells which will be helpful in providing appropriate diagnosis in difficult cases and provide better patient management.

KEYWORDS

Cell block (CB), Immunohistochemistry (IHC), Desmin (DES), Epithelial membrane antigen (EMA), Calretinin (CAL)

INTRODUCTION

The pleural mesothelium, derived from the mesoderm, is a monolayer of mesothelial cells that blanket the chest wall and lungs on the parietal and visceral surfaces, respectively. Various pathological processes such as inflammation, neoplasia, and trauma lead to reactive changes in the extremely sensitive mesothelial cells lining the serosal cavities. Mesothelial cell hypertrophy and proliferation in response to an altered environment may lead to a remarkably wide morphological spectrum, which may even overlap with malignant cells. Such exfoliated mesothelial cells with a wide range of appearances are usually referred to as " reactive mesothelial cells".[1,2]

Malignant mesothelioma (MM), is histologically classified in three major morphological types (WHO, 2021): epithelioid, sarcomatoid and mixed (or biphasic). The epithelioid pattern is present in about 70% of mesotheliomas, while 25% is biphasic and 5% sarcomatoid.[3,4] The common cytomorphologic features of mesothelial cells in reactive effusion include increase in cellularity of a monomorphic cell population associated with papillary clusters. The cells are larger than quiescent mesothelium, with some prominence of nucleoli, regular chromatin pattern, and normal nuclear to cytoplasmic ratio. Marked cytologic atypia can also be seen in hyperplastic or reactive mesothelium. The common cytologic features of malignant mesothelioma (MM) cells are nuclear pleomorphism, macronucleoli, large cellular aggregates, papillary–like tissue fragments, and cell-in-cell engulfment, but MM cells can also be deceptively bland and indistinguishable from benign mesothelial cells.[5]

Effusions in pleural and peritoneal cavities is a common complication of malignancies such as lung, breast, gastrointestinal and female genital adenocarcinoma as well as malignant mesothelioma.[6] Metastatic carcinomas are the most common tumors found in effusions. Of these, adenocarcinoma are more common than squamous and small cell carcinoma. The cells of metastatic carcinoma are larger, more pleomorphic, and more hyperchromatic than mesothelial cells. Adenocarcinomas exfoliate as large spheres or as isolated cells. Cytoplasmic vacuoles are often present, but they are not specific for adenocarcinoma; they are seen in some mesotheliomas as well as others tumors.[7] Effusions are a diagnostic challenge for the cytopathologist due to difficulty in distinguishing atypical mesothelial hyperplasia, mesotheliomas and metastatic adenocarcinoma.[8] To solve this problem, different methods are available including flow cytometry, DNA ploidy, telomerase activity and immunohistochemistry (IHC), but none showed reproducible result.[9] However, IHC is valuable for differential diagnosis. International Mesothelioma Panel recommended using at least two mesothelial markers and two carcinoma related markers to distinguished malignant mesothelioma from metastatic adenocarcinoma.[10]

Desmin, the intermediate filament protein is a known marker for smooth and skeletal muscle differentiation. Several studies have reported positive staining of Desmin in benign mesothelial cells (reactive mesothelial proliferation) in serous fluid and tissue sections.[9] Epithelial membrane antigen (EMA) is an antibody to a human milk fat globule membrane immunogenhigh molecular weight transmembrane glycoprotein expressed in cancer cells that suppresses cellular aggregation and cell matrix adhesion and promotes invasion of cellular matrix by malignant cells.[11] EMA staining with intense membranous pattern is highly suggestive of mesothelioma. EMA is expressed both in Malignant Mesothelioma (MM) and Metastatic adenocarcinoma (MA), appears to be the most useful marker in distinguishing benign from malignant mesothelial proliferations since EMA appears to be preferentially expressed in neoplastic mesothelium.[12]

Calretinin (CAL) is a 29-KD calcium binding protein that is normally expressed normally in neurons of central and peripheral nervous system.[13] CAL is both a sensitive and specific marker of reactive and neoplastic mesothelial cells in cytologic cell block preparations. It is found to be useful in differential diagnosis of Malignant mesothelioma and Metastatic Adenocarcinoma in malignant effusions.[14] Calretinin is a selective marker of mesothelioma of epithelial type and is absent from lung adenocarcinoma.

Desmin and EMA appear to be the most useful markers in distinguishing benign from malignant mesothelial proliferations. Desmin appear to be promising marker for the distinction between reactive mesothelium and malignant epithelial cells in terms of both specificity and sensitivity and its complementary use with calretinin is recommended. Desmin is preferentially expressed in reactive mesothelium and EMA in neoplastic mesothelium .[11,16] The current study is being taken up to compare the expression and efficacy of these proliferative markers in distinguishing benign and malignant proliferations in the workup of problematic pleural effusion in patients of this region to establish the importance of immunohistochemistry.

MATERIALS AND METHODS

A cross-sectional study spanning 2 years from October 2016 to September 2018 was conducted in the Histopathology section,

Department of Pathology, RIMS, Imphal. All pleural fluid samples and cell block of pleural fluid received in the Department of Pathology, during the study period were included in the study. Known cases of pulmonary T.B and malignancy under treatment, pleural fluid on routine examination showing only inflammatory cells and patients who didn't give written consent were excluded from the study. Socio-demographic & smoking habits of patient were recorded in the proforma prepared for the study. All together 46 pleural fluid samples were received which were grossly examined, smears was made, air dried and then it was fixed with 95% methyl alcohol and stained with routine May Grunwald Giemsa (MGG). Paraffin embedded cell block were prepared, H & E stain and IHC studies for Desmin, Epithelial Membrane Antigen and Calretinin was done.

Desmin (D33 Dako), Epithelial Membrane Antigen (E29 Dako) and Calretinin (Monoclonal Dako) were used in all IHC analysis. Sections of 3-5 µm were cut on to poly L-lysine coated slides and were deparaffinized and rehydrated. Antigen retrieval was achieved by microwave method using Tris buffer. Slides were allowed to cool for 20 minutes and blocking reagent was applied and kept for 10 minutes. Respective slides were covered with primary antibody (monoclonal Antibody EMA, Desmin and polyclonal antibody Calretenin) and were incubated for 1 hour at room temperature in humidity chamber. Polymer HRP labelled secondary antibody detection kit was added on the sections and incubated in humidity chamber for 30 minutes. DAB chromogen was added on the section for 10 minutes and then washed with D/W. All slides were counterstained with Hematoxylin, dehydrated and mounted. Between each step, the slides were washed with phosphate buffer solution (PBS).

Ethical approval was obtained from the Research Ethics Board, RIMS, Imphal with reference number A/206/REB-Comm(SP)/RIMS/2015/206/74/2016, dated 14th March 2018.and consent was obtained from each patient before the procedure. Data collected were kept secured.

Data collected were entered and analyzed using IBM SPSS Statistics 21 for Windows. Descriptive statistics like mean, standard deviation, percentages were used for variables like age, sex, occupation, religion, smoking, HPE and IHC findings. etc. Statistical analysis was done using Independent Samples t- test, ANOVA and ^{2} – test, p-values of < 0.05, < 0.01 and < 0.001 were taken as the cut off values for significance, highly significance and very highly significance respectively at 95% confidence interval.

RESULTS

Out of the 46 cases studied, 11 (23.9%) benign, 22 (47.8%) reactive, 7 suspicious for malignancy and 6 (15.2%) malignancy were diagnosed by cytological smear. Cell block H&E study showed 14 (30.4%) adenocarcinoma cases, 9 (19.6%) benign and 23 (50%) reactive. Final diagnosis was given according to H&E, pattern, intensity and proportion (PRO) of IHC staining where 11 (23.9%) turned out benign, 17 (37.0%) cases reactive, 16 (34.8%) adenocarcinoma and 2 (4.3%) cases were mesothelioma. Most of the cases were female 28 (60.9%) and 18 (39.1%) male, M: F ratio of 1:1.5 with an age range from 34 to 80 years (Mean±SE, 60.32±12.13) . Reactive was most common in older age (mean age 61.17 years) followed by benign (60.36 years), adenocarcinoma (60.06 years); and the youngest group was mesothelioma (55.00 years). All patients were married , (78.3%) were Hindus followed by Christian (17.4%%) and lowest was Muslim (4.3%). House wife and laborer were the commonness occupations with the respective percentages of 34.8% and 30.4% and next were businessman (15.2%), service holder (13.0%) and laborer (6.5%). 16 (34.8%) were smokers and 30 (65.2%) non-smokers (Table 1). Most of the lesions were unilateral 41 (89.1%) while bilateral was 5 (10.9%). Grossly 23 (50%) of the samples were reddish, 15 (32.6%) yellowish and 8 (17.4%) whitish, most of the adenocarcinoma 12 (52.2%) and mesothelioma 2 (8.7%) cases being reddish (Table 2).

Table 1: Socio-demographic & smoking characters of patients (N=46).

Parameters		No. of case	Percent
Sex	Female	28	60.9
	Male	18	39.1
Religion	Christian	8	17.4
	Hindu	36	78.3
	Muslim	2	4.3
Occupation	Business	7	15.2
	Farmer	14	30.4
	House wife	16	34.8
	Laborer	3	6.5
	Service holder	6	13.0
Marital status	Married	46	100.0
Smoking	No	30	65.2
	Yes	16	34.8
Total		46	100.0

Table 2 : Site & gross finding

Parameters		No. of case	Percent
Site	Bilateral	5	10.9
	Unilateral	41	89.1
Gross	Reddish	23	50.0
	Yellowish	15	32.6
	Whitish	8	17.4
Total		46	100.0

16 (94.1%) of the adenocarcinoma cases and 1 (5.9%) reactive case showed positivity for EMA Both the benign and reactive cases were negative for EMA. Desmin was positive in 8 (28.6%) of benign cases, 16 (57.1%) of reactive, 3 (10.7%) of adenocarcinoma and 1 (3.6%) of mesothelioma. Calretinin was positive in 2 (33.3%) of reactive , 3 (50.0%) adenocarcinoma and 1 (16.7%) mesothelioma case. However, all the benign cases were calretinin negative.

On statistical analysis , the test values suggest that there is strong association of IHC EMA, IHC DES, PRO EMA, PRO DES and PRO CAL with the final diagnosis as their respective p-values (<.001) was highly significant. However, IHC CAL didn't have significant association with the final diagnosis (p-value >0.05). (Table 3). Socio-demographic characteristic of the patients like sex, religion, occupation and smoking habit had no significant association with the development of malignancy (p-value >0.05). (Table 4). No association between age of the patients and development of cancer could be documented in our study. The site of lesion and gross finding was also insignificant (p-value >0.05). Out of 5 out of the 33 benign cases by cytology, turned out to be malignant on final diagnosis. (Cell Block H & E and IHC) and all 13 malignant cytology cases turned out malignant on final diagnosis. 4 out of the 32 benign cases of Cell Block H & E turned out malignant on final diagnosis while all 14 out of 14 malignant cases were confirmed for malignancy on final diagnosis. The sensitivity, specificity, positive predictive value and negative predictive value of Cytological diagnosis and Cell Block H & E were calculated (Table 5). Thus, from this interpretative analysis, one may conclude that Cell Block H & E is the superior diagnosis than Cytological diagnosis to detect malignancy through pleural effusion fluids specimens.

Table 3. Final diagnosis-wise distribution of cases according to IHC staining

Parameters		Final diagnosis				Total	x2-value	Df	P-Value
		Benign	Reactive	Adenocarcinoma	Mesothelioma				
IHC EMA	Positive	0	1(5.9%)	16(94.1%)	0	17(100.0%)	41.960	3	<.001
	Negative	11(37.9%)	16(55.2%)	0	2(6.9%)	29(100.0%)			
IHC DES	Positive	8(28.6%)	16(57.1%)	3(10.7%)	1(3.6%)	28(100.0%)	20.556	3	<.001
	Negative	3(16.7%)	1(5.6%)	13(72.2%)	1(5.6%)	18(100.0%)			
IHC CAL	Positive	0	2(33.3%)	3(50.0%)	1(16.7%)	6(100.0%)	4.542	3	.209
	Negative	11(27.5%)	15(37.5%)	13(32.5%)	1(2.5%)	40(100.0%)			
PRO EMA	0 - 20%	11(36.7%)	16(53.3%)	1(3.3%)	2(6.7%)	30(100.0%)	37.718	3	<.001
	> 20%	0	1(6.3%)	15(93.8%)	0	16(100.0%)			
PRO DES	0 - 20%	3(15.8%)	1(5.3%)	14(73.7%)	1(5.3%)	19(100.0%)	23.838	3	<.001
	> 20%	8(29.6%)	16(59.3%)	2(7.4%)	1(3.7%)	27(100.0%)			

PROCAL	0 - 20%	11(25.0%)	16(36.4%)	16(36.4%)	1(2.3%)	44(100.0%)	11.346	3	.010
	> 20%	0	1(50.0%)	0	1(50.0%)	2(100.0%)			
Total		11(23.9%)	17(37.0%)	16(34.8%)	2(4.3%)	46(100.0%)			

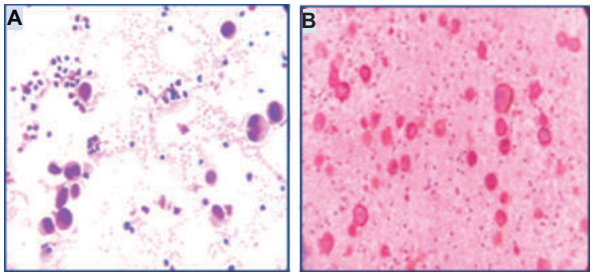
PRO : Proportion ; Df ; degree of freedom

Table 4: Final diagnosis-wise distribution of cases according to socio-demographic & smoking

Parameters		Final diagnosis				Total	x2-value	df	P-value
		Benign	Reactive	Adenocarcinoma	Mesothelioma				
Sex	Female	8(28.6%)	8 (28.6%)	11(39.3%)	1(3.6%)	28(100.0%)	2.527	3	.470
	Male	3(16.7%)	9(50.0%)	5(27.8%)	1(5.6%)	18(100.0%)			
Religion	Christian	2(25.0%)	2(25.0%)	4(50.0%)	-	8(100.0%)	4.773	6	.573
	Hindu	9(25.0%)	13(36.1%)	12(33.3%)	2(5.6%)	36(100.0%)			
	Muslim	-	2(100.0%)	-	-	2(100.0%)			
Occupation	Business	1(14.3%)	4(57.1%)	2(28.6%)	-	7(100.0%)	15.885	12	.197
	Farmer	3(21.4%)	2(14.3%)	7(50.0%)	2(14.3%)	14(100.0%)			
	Housewife	5(31.3%)	7(43.8%)	4(25.0%)	-	16(100.0%)			
	Labourer	-	3(100.0%)	-	-	3(100.0%)			
	Serviceholder	2(33.3%)	1(16.7%)	3(50.0%)	-	6(100.0%)			
Smoking	No	7(23.3%)	10(33.3%)	12(40.0%)	1(3.3%)	30 (100.0%)	1.198	3	.754
	Yes	4(25.0%)	7(43.8%)	4(25.0%)	1(6.3%)	16(100.0%)			
Total		11(23.9%)	17(37.0%)	16(34.8%)	2(4.3%)	46(100.0%)			

Table 5: Comparison of validity between Cytological diagnosis and Cell Block histomorphology

Type of test	CYTO diagnosis	CB H & E
Sensitivity	72.22%	77.77%
Specificity	100%	100%
Positive predictive value	100%	100%
Negative predictive value	84.84%	87.5%



A Fig.1: - (A) Pleural fluid smear showing reactive mesothelial cells. MGG stain, 40X , (B) Cell block pleural fluid showing desmin positive mesothelial cells. IHC stain for Desmin, 40X, Dako

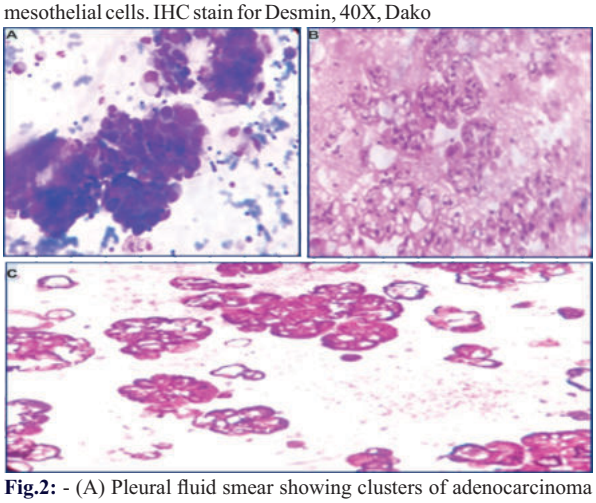


Fig.2: - (A) Pleural fluid smear showing clusters of adenocarcinoma cells. MGG stain, 40X., (B) cell block,pleural fluid showing malignant epithelial cells in sheets. H&E stain, 40X. (C) cell block,pleural fluid showing EMA positivity in malignant epithelial cells. IHC stain for EMA, 40X, Dako

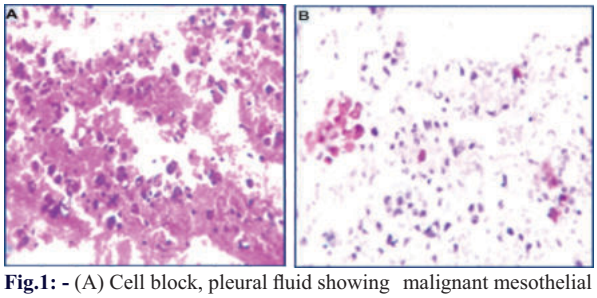


Fig.1: - (A) Cell block, pleural fluid showing malignant mesothelial cells.,H & E Stain, 40X., (B) cell block, pleural fluid showing Calretininin positivity in malignant mesothelial cells. IHC stain for Calretinin 40X, Dako

DISCUSSION

In the present study, the age range from 34-80 years with average of 60.32 years which was similar to the study conducted by Su XY et al16 where average age was 62 years with a range 30 to 82 years and Yahya ZM et al17 where majority of patients were 56±2 years with a range of 24 to 75 years. Most of the effusions were seen in 50-70 years thus projecting maximum number of cases in the sixth decade which is similar to findings of Nautiyal N et al18 and Bharath et al19 shown in between 50-59 years.

18(39.1%) cases were male and 28(60.9%) female patients with a M:F ratio of 1:1.5 which is comparable to the study conducted by Dey S et al20 which showed M:F ratio of 1:1.7. Studies by Ensani F et al21 and Su XY et al16 also showed a similar M: F ratio of 1: 4.9 and 0.79:1 respectively. As can be noted, malignant pleural effusions are more likely in females owing to increase number of cases of lung carcinoma in females.

The nature of the pleural fluids in our study were described as reddish/haemorrhagic in 23(50.0%) samples, yellowish in 15(32.6%) and whitish in 8(17.4%) samples with the most common cause of haemorrhagic effusions being malignancy which was present in 14(60.9%). This is comparable with the findings of study by Yahya ZM et al.17

Sensitivity and specificity of cytological (smear) examination were found to be 72.22% and 100% . In the study by He DN et al22 sensitivity was 40-60%. When CB procedure was compared to conventional smear in terms of preservation of architectural pattern (i.e., presence of three-dimensional cell clusters, cells balls, papillae, glandular or acinar formations, sheets, it was found that cytoarchitectural pattern was far more well-preserved in cell block specimens (77.77%) than in conventional smear (72.22%) which is comparable to the findings of Dey S et al20 where cell block procedure have high sensitivity (88.88%).

Of the 46 cell blocks, 18 cases were histologically positive for malignancy of which 16/18 cases showed strong membranous and cytoplasmic positivity for EMA with 2/18 cases showing strong nuclear and cytoplasmic positivity for CAL. Diffuse cytoplasmic staining of Desmin was detected in 28 cases of pleural fluids of which 59.3% are reactive mesothelial cells and 3.7% of malignant mesothelioma (MM) cases which was statistically highly significant (p□0.001). Parallel to such results, Al Mehy GF et al23 reported that Desmin was positive in 88.2% of cases of reactive mesothelial cells and 7.7% of MM cases (p□0.001). Also, Hasteh et al5 found that strong cytoplasmic staining for Desmin is observed in 84% of cases of reactive mesothelial cells and 6% of MM cases.

In the current study, EMA expression was detected in 6.3% of reactive mesothelial cells proliferations and 93.8% malignant epithelial cells were EMA positive with strong membranous accentuation and cytoplasmic staining. This was statistically highly significant (p□0.001). These results were in agreement with findings by Al mehy GF et a23 where EMA was positive in 5.9% of reactive mesothelial cells and all cases of atypical mesothelial cells (p□0.001). Also, Hasteh et al5 also observed that EMA is positive in 9% of benign reactive mesothelial cells.

This study suggested that EMA and Desmin serve as a markers for mesothelial cells in the appropriate setting and their combination can aid in distinction between reactive and malignant mesothelial and malignant epithelial cells. These findings are concordant with the results of Attanoos et al.24

Using Calretinin in this study , one (50%) of malignant mesothelioma case and one case of reactive mesothelial cells have shown a positive nuclear reaction similar to results by Politi et al25. 11 (25.0%) benign cases and 16 (36.4%) reactive mesothelial cells show weak positivity staining with CAL, which is contrary to the findings by Murugan P et al10 as they observed CAL staining in all cases of reactive mesothelial cells. The presence of faintly CAL- positive (□20%) adenocarcinomas in this study was only 3 cases. The staining pattern of cells of mesothelial origin was cytoplasmic with peripheral condensation and accompanying nuclear staining. Similar to the observations made by Chhieng DC et al13, the staining of adenocarcinoma cells with CAL was focal and less intense when compared to that of reactive and neoplastic mesothelial cells.

The conflicting results of CAL expression in adenocarcinoma and malignant mesothelioma with no significant association in our study could be attributed to differences in patients population studied, data analysis, the scoring system, duration and condition of incubation, different immunoperoxidase detection system, variation in the evaluation of immunostaining results and/or staining techniques using different antibodies and antigen retrieval methods.

The combined use of cytology, cell block H&E and IHC were evaluated in the current study and thus by combining the results of this techniques produced an increase in sensitivity and accuracy of diagnoses, particularly in malignant effusions.

CONCLUSION

In this study, IHC expressions of EMA, Desmin and Calretinin were studied in 46 pleural effusions which helped in confirming the adenocarcinoma cells or reactive and malignant mesothelial cells. Combination of positive EMA and negative DES correctly identified 15 of 16 (93.8%) cases of adenocarcinoma, whereas the combination of negative EMA and positive DES was seen in 16 of 17 (59.3%) cases of reactive mesothelial cells. However, for typing of adenocarcinoma an additional panel of immunomarkers has to be employed. This study suggest an important role for Desmin as a differentiating marker between reactive and malignant mesothelial cells. Desmin might therefore be an ideal complementary marker of mesothelial cells together with Calretinin. Calretinin expression can be a very useful marker for the distinction between Adenocarcinoma and mesothelial lineage both reactive and neoplastic. 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 complimentary use of both markers is advocated in ascertaining the nature of mesothelial proliferations. This study confirms the usefulness of these IHC markers in distinguishing between reactive and malignant epithelial cells with malignant mesothelial cells when stromal invasion are difficult to assess and may help in accurate diagnosis of difficult cases.

CONFLICT OF INTEREST

There are no conflicts of interest.

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