Use of Panel of Markers in Serous Effusion to Distinguish Reactive Mesothelial Cells from Adenocarcinoma

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Abstract

INTRODUCTION: Although cytological examination helps in diagnosis of malignancy in serous effusion, at times it is difficult to differentiate atypical reactive mesothelial cells from adenocarcinoma (AC) cells. To resolve this problem, various ancillary methods have been used. Immunocytochemistry (ICC) is one such commonly used technique in which various panel of antibodies has been tried. Unfortunately, so far no unique marker is available to solve this issue. Hence, the present study evaluates the efficacy of four antibody panel comprising of MOC-31, epithelial membrane antigen (EMA), calretinin (CAL), and mesothelin (MES) to solve this problem. MATERIALS AND METHODS: Forty-two cases suspected of malignant effusion in pleural/peritoneal fluid and 42 cases of reactive effusion were included. Cytospin smears were prepared and stained with Giemsa stain for cytomorphological diagnosis. Cytospin smears and cell blocks were made for ICC. ICC for MOC-31, EMA, CAL, and MES was performed. RESULTS: Among the suspected malignant effusion cases, 30 cases were AC and 12 cases were suspicious for malignancy by cytomorphology. MOC31 demonstrated 100% sensitivity (Sn) and 95.24% specificity (Sp), and EMA had 88.1% Sn and 92.86% Sp for AC cases. CAL demonstrated 100% and 97.62%, and MES 97.62% and 88.1% Sn and Sp in reactive mesothelial cells, respectively. CONCLUSION: In conclusion, combination of MOC-31 and CAL as a limited panel will be helpful in giving an appropriate diagnosis in difficult cases and thereby, help in patient management. In addition, ICC on cytospin smears gave results similar to cell blocks, and if standardised cytospin is simple technique to perform, unlike cell blocks.

Keywords: Adenocarcinoma, calretinin, cell block, immunocytochemistry, MOC-31, reactive mesothelial cells

INTRODUCTION

Cytological examination of body fluid is useful to detect malignancy, but a definitive diagnosis cannot always be made on cytologic evaluation alone when it is admixed with reactive mesothelial cells or mesothelioma where the cells mimic adenocarcinoma (AC) cells. Mesothelial cells because of the wide variety of stimuli and injuries that break their continuity show reactive changes such as proliferation and cellular changes including marked nuclear and cytoplasmic alterations that can mimic the morphology of malignant cells.¹,² Thus, atypical reactive mesothelial cells serve as a major pitfall for a false positive diagnosis of malignancy. In treated cancer patients, effusion often represents the first manifestation of recurrent disease. Sometimes, low-grade carcinoma can masquerade as benign ones. Benign mesothelium undergoes myriad architectural, and cellular alterations in reaction to numerous stimuli, while, well differentiated or borderline malignant cells can masquerade as benign ones.³ Hence, it is important to identify malignant cells in effusion samples for therapeutic and prognostic purpose. In such cases, ancillary diagnostic techniques are needed to solve the dispute. Immunocytochemistry (ICC) analysis is one such easily performed technique and uses a panel of markers.⁴⁻¹⁰ However, there is a huge lacuna in achieving an accurate panel of immunomarkers as a diagnostic aid in solving the problems. Several antibodies have been tried in the differentiation of reactive mesothelial (RM) cells from metastatic ACs; unfortunately, no single marker is so far 100% specific and sensitive for neither AC cells nor RM cells.¹⁴⁻¹⁸ Hence, the present study evaluated the efficacy of four markers.
that include MOC-31, epithelial membrane antigen (EMA), calretinin (CAL), and mesothelin (MES) to differentiate AC cells from RM cells in serous effusions.

**Materials and Methods**

Forty-two cases suspected of malignant effusions of pleural and peritoneal fluid and positive for malignant cells on cytomorphology over a period of one year were included in the study. Forty-two reactive effusion cases were included as control samples. Cytologically, effusion with inflammatory cells without mesothelial cells or malignant cells were excluded. In all cases, cytospin smears were prepared using Shandon Cytospin-3 technique.

Cytoslides, filter card, and cytofunnels were assembled in the cytoclip, and cytoclip was fitted into the Shandon machine. Then, 0.5 ml of fluid was added into the funnel, and machine was run for 10 min at 2000 rpm. Minimum of two slides were prepared and stained with Giemsa for morphological diagnosis.

Extra four smears were prepared wherever possible for ICC. The extra smears were wrapped in aluminium foil and preserved at 0°C in refrigerator for ICC. The cell blocks (CBs) were prepared where the adequate samples were available by using Thermo Shandon cell block kit.

ICC was performed on 18 cases taken as CB sections, 12 cases as fresh smears, and 12 cases as both smears and CB sections. Among the control cases, ICC was done on 28 cases as smears, 8 cases as CBs, and 6 cases as both CBs and smears.

Primary antibodies used were EMA (mouse monoclonal antibody GP 1.4, DBS), MOC-31 (MOC-31, Bio SB), CAL (DAK-Calret 1, Dako), and MES (HBME1, Dako). BioGenex Super Sensitive Polymer-HPR Detection System—a biotin free detection system was used in conjunction with rabbit/mouse IgG primary antibodies. Diaminobenzidine as a substrate for peroxidise, which was used as an enzyme label. Citrate buffer was freshly prepared each time and used for antigen retrieval. Positive and negative controls for all the antibodies were run each time.

Smears were fixed in a mixture of acetone and methanol (50:50) (20°C) for 5 min.

The two authors independently evaluated the slides. Staining in more than 20% of cells was considered as significant staining and positive.

Statistical analysis was done using Chi-square test and Fisher’s exact test using SPSS software version 16.

**Results**

Among the 42 AC cases, 30 (71.43%) cases showed definitive evidence of malignancy, and 12 (28.57%) cases were given as suspicious for malignancy. Sensitivity (Sn) for definitive diagnosis of AC according to cytomorphology alone was 71.43%. Out of 42 cases, 15 cases were pleural effusion, and 27 cases were peritoneal effusion. Primary site of carcinoma were ovary (29 cases), lung (7 cases), gastrointestinal tract (4 cases), pancreas, and unknown primary (1 case each). All the cases were proven malignancy by histopathology, where in the unknown primary on further evaluation was found out to be colonic AC. We did not encounter any case of mesothelioma.

Definitive adenocarcinomatous cases, cytologically were seen as cells arranged in large cellular aggregates, papillary clusters, acinar pattern [Figure 1a, a-inset], and signet ring cells. In 12 cases, definitive morphological diagnosis was not possible; in these cases, atypical cells were seen scattered with nuclear atypia, signet ring cells along with RM cells. All the cases on ICC confirmed malignancy.

Among control cases, RM cells were seen as papillary forms, sheets, and scattered cells with binucleation [Figure 1b, b-inset]. All theses cases had mixed inflammatory infiltrate in the background.

The results of ICC are shown in Tables 1 and 2.

MOC-31 was expressed in 100% of all the AC cases including the suspicious cases, out of which 35 (83.33%) cases showed strong membranous pattern, while 7 (16.67%) cases exhibited membranous accentuation with cytoplasmic staining [Figure 2a]. In RM cells, MOC-31 was expressed in 2 (4.8%) cases both of them showed weak cytoplasmic staining pattern.

EMA was expressed in 37 (88%) of 42 AC cases. In 33 cases, it showed strong membranous accentuation pattern [Figure 2b], while in 4 cases there was strong cytoplasmic staining with membranous accentuation. Among the 42 reactive cases, it showed weak membranous staining in 3 (7.1%) cases.

In all RM cases, CAL showed 100% Sensitivity (Sn) with nuclear and cytoplasmic staining pattern or nuclear staining background.

The extra smears were wrapped in aluminium foil and preserved at 0°C in refrigerator for ICC. The cell blocks (CBs) were prepared where the adequate samples were available by using Thermo Shandon cell block kit.

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MES showed strong membranous staining in 41 (97.6%) of 42 RM cases [Figure 2d]; while in AC cases, it was expressed in 5 (11.9%) cases. Out of these 5 AC cases, 4 cases showed strong cytoplasmic staining pattern, one case showed focal membranous staining pattern.

Thus, MOC-31 was the overall single best marker for the AC cells, exhibiting 100% Sn and Sp (95.24% Sp). EMA also exhibited high Sn and Sp, but slightly on lower side compared to MOC-31. Among the RM group, CAL had 100% Sn and 97.62% Sp. MES also had high Sn of 97.62%, but its low Sp (88.10%) limits its use as a sole mesothelial marker.

Table 1: Overall results of ICC

|          | MOC-31 | EMA     | CAL     | MES     |
|----------|--------|---------|---------|---------|
| AC (n=42)| 42 (100%) | 37 (88%) | 1 (2.4%) | 5 (11.9%) |
| RM (n=42)| 2 (4.8%)  | 3 (7.1%)  | 42 (100%) | 41 (97.6%) |
| Sp       | 95.24% | 92.86%  | 97.62%  | 88.10%  |
| Sn       | 100%   | 88.1%   | 100%    | 97.62%  |
| PPV      | 95.45% | 92.5%   | 97.67%  | 89.13%  |
| NPV      | 100%   | 88.64%  | 100%    | 97.37%  |
| LR       | 21.00  | 12.34   | 42.01   | 8.20    |
| $\chi^2$ | 76.36 | 55.17   | 80.09   | 62.28   |
| $P$      | <0.0001| <0.0001 | <0.0001 | <0.0001 |

AC: Adenocarcinoma, RM: Reactive mesothelial, n: Number of cases, EMA: Epithelial membrane antigen, CAL: Calretinin, MES: Mesothelin, Sp: Specificity, Sn: Sensitivity, PPV: Positive predictive value, NPV: Negative predictive value, LR: Likelihood ratio

Table 2: Results of ICC in suspicious cases

|          | MOC-31 | EMA     | CAL     | MES     |
|----------|--------|---------|---------|---------|
| AC (n=12)| 12 (100%) | 12 (100%) | 0 (0%)  | 1 (8.3%) |
| Sn       | 100%   | 100%    | 0%      | 8.3%    |

Figure 2: ICC: a. MOC-31 in cytospin smears. Adenocarcinoma clusters showing strong membranous and cytoplasmic positivity. (x200). b. ICC on cell block EMA showing strong membranous positivity in adenocarcinoma cells. (x200). c. Calretinin in cytospin smears showing strong nuclear and cytoplasmic positivity in reactive mesothelial cells. Background inflammatory cells are negative. (x200). d. Mesothelin in cytospin smears showing strong membranous positivity in mesothelial cells. (x200)

**Discussion**

Cytological examination is a routine method for the diagnosis of malignant effusions. However, one cannot rely on morphological diagnosis completely. The present study examined the cytomorphology and efficacy of ICC in suspicious for malignant effusion, which also states a Sn of 71.43% for cytological diagnosis. Similar findings were observed by various studies with average Sn for conventional cytological diagnosis as 58%.[12-3]

CB is a simple technique and has been shown to be useful adjunct to centrifuged samples of effusion smears for a more definitive cytological diagnosis, particularly where morphology is not possible with smears alone.[19] Sections from the CB showed optimum morphology that was almost parallel to histopathology in some cases. Not only does CB preserved cellular architecture, but it can also be used for special stains and immunohistochemistry. Additionally, unlike smears, CBs can be preserved for a longer period of time without losing the antigenic properties of the cells. Thus, sections from CB could supplement cytospin smears for ICC and definitive diagnosis in equivocal cases. The present study analyzed both CB preparations and cytospin preparations for immunostaining and found both types of preparations to be comparable with regard to the quality of immunoreactivity.

In recent years, ICC analysis has contributed greatly to differentiate between AC and mesothelial cells. Most of the available markers, such as carcinoembryonic antigen (CEA), CD15 (Leu M1), BerEP4, and B72.3, recognize molecules commonly expressed by ACs but not by mesothelial cells.[6,9,11] However, the expression of these markers were not uniform among different types of ACs and also not diagnostic of primary site of origin.

Various panels have been tried over a decade. Studies done earlier prefer 2 epithelial and 2 mesothelial markers to solve this task.[4,6-9]

In our study, MOC-31 showed strong membranous staining in all AC cases including the suspected ones, where in tumor cells showed intense staining with negative mesothelial markers. In the present study, two cases (4.8%) of RM cells expressed focal cytoplasmic staining; however, typical membranous accentuation was not seen as compared to the AC cells. Kundu et al.,[8] Lozano et al.,[6] and Hecht et al.[17] also found MOC-31 expression in RM cells.

Although EMA also showed strong membranous or cytoplasmic with membranous accentuation staining in AC cells, EMA was also expressed in few RM cells, with weak membranous staining pattern. This result is comparable with findings from other studies done earlier that also observed weak expression of EMA in normal or RM cells and strong expression in mesothelioma.[4,9,20,21] Because of this difficulty in interpretation of staining patterns, we conclude MOC-31 is preferred over EMA for AC cases. Moreover, our study included variety of primary sites and all cases expressed same intensity.
Among the RM group, we observed 100% Sn for CAL, with nuclear and cytoplasmic staining or nuclear positivity. Hence, in this study, we conclude intense nuclear staining should be considered as positive. The reported incidence of CAL positive in ACs ranged from 5–10% including colonic and ovarian ACs.[4,8,10-12] In the current study, one ovarian AC (2.4%) showed positivity for CAL; the staining pattern was focal, weak, and less intense when compared with that of RM cells; and similar to previous studies.[8,10]

In the present study, MES was expressed in all except one RM cases with strong membranous staining pattern with Sn of 97.62%. Among AC cases, 5 cases expressed this antibody, which included ovarian AC (3), lung, and pancreatic AC (1 case each). Ordonez et al.[14] and Yaziji et al.[13] found apart from mesothelial cells this antibody was also expressed in ovary, pancreas, and lung ACs. However, the staining pattern differs between these two; strong membranous pattern favors RM cells, cytoplasmic, mixed cytoplasmic, membranous, and focal membranous staining pattern favors AC.

Among the 12 suspicious for malignant effusion cases, both MOC-31 and EMA showed 100% Sn, whereas CAL was negative in the same cell clusters. The background RM cells stained positive for CAL. However, in one case (primary site ovary) MES stained RM cells as well as suspicious AC cells, but the staining pattern differed in these two cell types.

During our one year study period, we did not come across any case of mesothelioma. However, the differentiation between RM cells and mesothelioma would be according to the cytological features as the existing mesothelial markers are helpful in identifying whether the cell is of mesothelial origin, rather than differentiate RM cells from cells of malignant mesothelioma.

Until now, no single marker is capable of differentiating RM cells from AC cells. The present study also favors a panel of antibodies to arrive a definitive diagnosis as inferred by others. Thus, we conclude ICC markers MOC-31 and CAL as a limited panel will be helpful in differentiating AC cells from RM cells in cytologically difficult cases. It can be performed both on cytospin smears as well as on CBs, with similar intensity in results, where in by smear technique would be easy and time saving.

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Conflicts of interest
There are no conflicts of interest.

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