Utility of a limited panel of calretinin and Ber-EP4 immunocytochemistry on cytospin preparation of serous effusions: A cost-effective measure in resource-limited settings

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Abstract

Background: Differentiation between reactive, but morphologically atypical, mesothelial cells and adenocarcinoma in effusions can be problematic. Elaborate immunohistochemical panels have been devised. Techniques like DNA analysis, flow/image cytometry, and K-ras mutation analysis are research oriented and difficult to perform in routine, especially in resource-poor centers. We evaluated the efficacy of a limited two-antibody panel comprising calretinin and Ber-EP4 on cytospin and cell block preparations, in 100 effusion samples. Materials and Methods: Fifty cases of reactive mesothelial hyperplasia and 50 cases of adenocarcinoma diagnosed by cytomorphology in ascitic/pleural fluid specimens over a 2-year period were assessed. The diagnoses were confirmed by clinical/histopathologic correlation. Cytospin smears were made in all. Cell blocks were prepared, wherever adequate fluid was available. Immunocytochemistry (ICC) for calretinin and Ber-EP4 was performed. Results: Forty-five of the reactive effusion cases (90%) were calretinin reactive and Ber-EP4 negative. Among the adenocarcinoma cases, 49 (98%) were calretinin negative but Ber-EP4 positive. Thus, both calretinin and Ber-EP4 had a high sensitivity (90% and 98%, respectively), as well as a high specificity (100% and 86%, respectively). In the 21 reactive mesothelial cases, whose cell blocks were made, results were comparable to those on cytospin. However, of the 19 adenocarcinoma cases in which cell blocks were prepared, all were Ber-EP4 immunopositive except for three, which were positive on cytospin, implying false-negative results on cell blocks. Conclusions: A limited panel of two monoclonal antibodies, calretinin and Ber-EP4, may be useful in cytology, as a “primary antibody panel”, for accurate diagnosis and patient management. Additionally, ICC can be performed easily on cytospin preparations, which gave results comparable to cell blocks in our study.

Key words: Ascitic, Ber-EP4, calretinin, cytology, effusion, immunocytochemistry, immunohistochemistry, peritoneal, pleural

INTRODUCTION

Aspiration of serous cavity fluids (pleural, pericardial and peritoneal) has become a routine procedure for aiding in diagnosis. Among malignant tumors, involvement of serous membranes by secondary tumors is by far more common than primary malignant tumors of the mesothelium. Adenocarcinomas are the commonest tumors to show involvement of serous membranes. Most frequently encountered are tumors of the breast,
ovaries and lung, followed by gastrointestinal tract and the rest of genitourinary system.[11] Effusion may be the first presenting feature of malignancy in many patients.

Reactive mesothelial cells (RMCs) are invariably associated with most serous effusions. The cells are hypertrophic and hyperplastic and may mimic morphologic appearance of neoplastic cells.[12,13] It may not be possible to definitively distinguish RMCs from adenocarcinoma based on morphology alone and ancillary techniques may be required in such a situation, amongst which immunocytochemistry (ICC) is most often used. Most studies recommend an antibody panel comprising a combination of mesothelial and epithelial markers.[3-15] Murugan et al., in 2009, had reviewed the various panels of antibodies recommended after 1995.[14] Fetisch and Abati also reviewed various antibodies which may be used for the purpose.[16] However, application of an extensive antibody panel is not cost-effective and hence would not be feasible as a routine, especially in resource-poor countries.

Cell block sections are the preferred choice of cytologic preparation for immunostaining due to comparability with surgical material, absence of background staining and ability to assess the same pattern of cells in step sections. In addition, if required, multicolor-multiplex immunostaining may performed on cell block sections, which is not possible on cytology smears.[8,16-18] Despite these advantages of cell blocks, in centers with limited resources, cytospin preparations may offer a useful alternative for evaluating immunoprofile.

We attempted to differentiate RMC in ascitic/pleural fluid specimens from metastatic adenocarcinomas using a limited panel of two monoclonal antibodies, calretinin and Ber-EP4, and compared the results of ICC on cytospin smears with those on cell block sections.

**MATERIALS AND METHODS**

Consecutive samples of ascitic/pleural fluid submitted to the Cytopathology Laboratory, Department of Pathology, All India Institute of Medical sciences, between January 2006 and January 2007, with a clinical and/or radiological suspicion of malignancy were studied. Smears were prepared from each sample using routine centrifuge and cytocentrifuge. Remaining fluid was used for preparing cell blocks. Each sample was centrifuged at 1500 rpm for 10 minutes in four different tubes. Three alcohol-fixed and one air-dried smear were made from the sediment and subjected to Papanicolaou and May-Grünwald (MGG) staining. The slides were blindly reviewed by two cytopathologists (SM and VK) and a preliminary consensus diagnosis was reached. First 50 samples each of reactive mesothelial effusion and malignant effusion were included in the study. All the diagnoses were confirmed by clinical presentation/histopathology.

Cytospin preparations were also made using the cytocentrifuge (Thermo Shandon, Pittsburgh, PA, U.S.A.) at 1500 rpm for 10 minutes. Eight smears were prepared of which two were stained with Papanicolaou stain and MGG stain, respectively, for morphological assessment. Two were used for ICC using anti-calretinin and anti–Ber-EP4 antibodies, and two were kept aside for negative control for both the antibodies. Negative controls were applied with each batch of slides put up for ICC. The remaining two were used for repeat ICC, in case of unexpected and unsatisfactory results.

Formalin-fixed, paraffin-embedded cell blocks were prepared in all cases with adequate fluid available using Nathan alcohol formalin method.[19] Nathan alcohol formalin substitute (NAFS) consists of 1:9 parts of 40% formalin and 100% ethyl alcohol. NAFS was added to spun-down cell pellets after discarding the supernatant. The sample was recentrifuged after a minimum of 45 minutes and the cell pellet was then wrapped in a paper and is kept in cassette and stored in 80% ethanol. Then, paraffin-embedded cell blocks were prepared.

At least three slides comprising serial sections at 3–4 µm were obtained from these cell blocks. One of the slides was stained with Hematoxylin and Eosin (H&E) stain, while the other two slides were subjected to ICC for calretinin and Ber-EP4 using streptavidin–biotin peroxidase technique.

**Immunocytochemistry**

Monoclonal antibodies for calretinin (Novocastra, United Kingdom, Clone NCL-L-CALRETININ, dilution 1:100) and anti-human epithelial antigen (DakoCytomation, Glostrup, Denmark, Clone Ber-EP4, dilution 1:200) were used. Anti-human epithelial antigen will be referred by the name of Ber-EP4 in this article. Labeled streptavidin–biotin kit (Novocastra, United Kingdom) was used as detection system. Antigen retrieval for both was done by boiling in 0.01 M citrate buffer, pH 6.0. Purkinje cells of the normal cerebellum were used as positive control for calretinin, while ovarian adenocarcinoma was used for Ber-EP4. Negative controls were obtained by omitting the application of primary antibody during the immunostaining process and using TBS instead.

Calretinin and Ber-EP4 immunoreactivities were analyzed. A case was considered to be positive when 50% cells or more of the relevant cell population were immunoreactive. If only 10% or less of the cells were reactive, then the case was regarded as negative.[13] If in any case, 10–50% cells were immunopositive, the results were considered inconclusive and the tests were repeated. Calretinin stained both the nuclei and the cytoplasm of the cells. Ber-EP4 was regarded as positive when it stained both the cell membrane and the cytoplasm of the tumor cells. ICC was repeated if the slides were not interpretable as in the
case of high background staining, or if there was lack of correlation between the results on cytospin smears and on cell block preparations.

The cytopathologist (RA) reviewing the immunostained slides was blinded to the original cytology diagnosis.

RESULTS

Cytomorphological features

The cases morphologically classified as reactive mesothelial effusion (RMCH) showed RMCs present singly or more often in berry-like clusters with scalloped contours. There were no true papillary or acinar structures. Nuclei were round with smooth contours. The cells had low nuclear to cytoplasmic ratio. Cytoplasm was usually abundant and dense with peripheral fuzziness and occasional cytoplasmic vacuoles [Figure 1a and c]. In contrast, adenocarcinoma cells were seen singly or as groups with smooth contours and hard anatomical edges. Acinar structures, papillary structures, proliferation spheres and solid cell clusters were frequently seen [Figure 1e and g]. Nuclei were variably sized, often with irregular contour, prominent nucleoli and high nucleus to cytoplasmic ratio.

In 15 cases, it was not possible to impart a definitive diagnosis on morphology alone due to overlapping cytologic features and these were considered as inconclusive for involvement by carcinoma. These cases showed the presence of singly lying cells having abundant cytoplasm and peripheral fuzzy borders similar to mesothelial cells, but the nuclei demonstrated moderate to marked pleomorphism, hyperchromasia and prominent nucleoli [Figure 2a and c]. One of the cases showed cells arranged in tight clusters but bearing bland nuclear features [Figure 2e]. This case also, on histopathology, showed peritoneal deposits from an adenocarcinoma. Some of the cases showed only an occasional tight cluster of highly atypical cells in a background of reactive mesothelial proliferation [Figure 2g]. On review of corresponding histopathologic sections, 10 could be characterized as malignant effusions and 5 as RMCH.

Immunocytochemistry on cytospin preparations

Results of ICC performed in all the cases on cytospin preparations are depicted in Table 1.

Most (45/50; 90%) of the cases classified as RMCH on morphology were strongly and diffusely calretinin positive [Figure 1b]. Of these, seven cases also showed focal (less than 10% cells) and weak immunostaining with Ber-EP4. Replicate results were obtained when ICC was repeated for these cases. On review of cytospin preparations of these seven cases, no atypical cells could be identified on morphology, implying a false-positive Ber-EP4 reaction. The remaining five cases which were calretinin negative were also negative for Ber-EP4.

It was found that morphologically atypical cells in 49 of the 50 cases (98%) diagnosed initially as adenocarcinoma on morphology were positive for Ber-EP4 [Figure 1f]. There was only one case in which the tumor cells were negative for Ber-EP4. This case was also negative for calretinin. Repeat ICC yielded similar results. In all these cases, calretinin showed positivity in the background RMC, wherever present.

ICC was helpful in correctly diagnosing the 15 inconclusive cases. Five of these samples were immunopositive for calretinin and negative for Ber-EP4. The rest 10 showed Ber-EP4 immunoreactivity [Figure 2b 2f and 2h]. These results correlated well with the final histopathologic diagnoses.
Immunocytochemistry on cell blocks

Cell blocks were prepared in 21 RMCH cases and 19 cases of malignant effusion. ICC was performed on paraffin sections obtained from these blocks, the results of which are depicted in Table 2.

When the 21 cases of RMCH with available cell blocks were assessed, most (18/21; 85.71%) were calretinin positive, a finding similar to that on cytospin [Figure 1d]. Of these 18 cases, 2 also showed focal and weak Ber-EP4 staining. Similar results were seen on corresponding cytospin preparations, and were regarded as false positive. The three calretinin-negative cases were also negative for Ber-EP4.

Ber-EP4 was positive in majority of the cases (16/19; 84.21%) of malignant effusions [Figure 1h]. However, the three cases, which were negative on cell blocks, were strongly immunopositive for Ber-EP4 on cytospin slides. Repeated ICC performed on cell block and cytospin slides for these three cases gave replicate results. All 19 cases were negative for calretinin on both cell blocks and cytospin.

Of the 15 cases, in which a conclusive diagnosis could not be reached on cytomorphology alone, cell blocks were made in 10 cases and the results of ICC were similar to those on cytospin preparation [Figure 2d].

DISCUSSION

Effusion cytology is a commonly used modality to stage and even diagnose various malignancies. RMC can at times show cytologic features indistinguishable from cells shed from an adenocarcinoma, leading to erroneous diagnoses.\[1,2\]

Monoclonal antibodies against various mesothelial and epithelial antigens have been used to aid in differentiating the two. Over the past several years, various studies have analyzed the efficacy of a variety of antibodies for this purpose.\[3-15,20-34\] But till date, there has been no consensus on the panel of antibodies to be used. The problem of separating reactive mesothelial from neoplastic epithelial cells continues to challenge cytopathologists in routine practice. The general guideline of using a panel of four antibodies, comprising

![Figure 2: Pleural fluid showing singly lying atypical cells (a, cytospin- MGG x400; inset, cytospin- Papanicolaou x400). Staining for Calretinin was positive confirming their mesothelial nature (b, cytospin- ICC x400). Ascitic fluid sample of another case (c, cell block- H and E x400) with single atypical cells positive for BerEP4 (d, cell block- ICC x400) and negative for calretinin (d, inset, cell block- ICC x400). Bland appearing cell clusters from a case of metastatic adenocarcinoma (e, cytospin- Papanicolaou x400) positive for Ber EP4 (f, cytospin- ICC x400). Pleural fluid from a case of metastatic adenocarcinoma (g, cytospin- Papanicolaou x400) showing few atypical tight clusters. Positivity for calretinin indicated mesothelial proliferation (h, cytospin- ICC x400).](image-url)
two positives and two negatives with coordinate pattern, gives reliable results with tissue specimens. However, the same is not applicable for cytologic smears due to the intrinsic limitation that the same cell cannot be present in contrast to that with 3–4 µm serial adjacent histologic as well as cell block sections, especially when the neoplastic cells are scarce and scattered singly. In addition, cytologic smears also suffer with the limitation of inability to evaluate multiple immunomarkers simultaneously. Some authors have recommended single antibodies, mostly calretinin. We do not agree with the use of a single monoclonal antibody for this purpose, which has therapeutic as well as prognostic implications. This is in agreement with other authors. A minimum of two antibodies, a mesothelial and an epithelial marker, should be included as it may not be possible to use an exhaustive panel of antibodies each time, especially in a tertiary care center like ours, which deals with many patients every year. Keeping this in view, we analyzed two monoclonal antibodies, viz. calretinin (a mesothelial marker) and Ber-EP4 (an epithelial marker), on cytospin preparations from 50 cases of reactive mesothelial hyperplasia and 50 cases showing involvement of serous effusions by adenocarcinoma.

Most (45/50; 90%) of the 50 cases of RMCH showed strong calretinin reactivity. Of the cases diagnosed as adenocarcinoma on morphology, all but one showed Ber-EP4 immunopositivity. Interestingly, Ber-EP4 could highlight tumor cells in 10 of the morphologically ambiguous cases. In these, there was strong and diffuse reaction to Ber-EP4, along with absence of calretinin reactivity.

Calretinin stained the RMC, wherever present in the cases of malignant effusion, but was negative in the tumor cells.

Thus, both the antibodies, calretinin and Ber-EP4, had a high sensitivity (90% and 98%, respectively), as well as a high specificity (100% and 86%, respectively).

Sensitivity and specificity of different mesothelial and epithelial markers have been variable in different studies. Considering individual markers, Ber-EP4, MOC-31, epithelial membrane antigen (EMA) and calretinin appear to have the preferred combination of high sensitivity clubbed with high specificity of more than 90% each. But EMA shows distinctive staining patterns with malignant mesothelioma and with adenocarcinoma, being membranous in the former and diffuse cytoplasmic in the latter. In addition, it stains a small percentage of reactive cases also. Due to this difficulty in interpretation of staining patterns, it is not a preferred marker. Grefte and co-authors also evaluated six markers, three each

Table 3: A comparison of sensitivity and specificity of the various epithelial and mesothelial markers

| Author Ref. | Preparation | SN/SP | B72.3 (%) | Ber-EP4 (%) | MOC-31 (%) | EMA (%) | E-cad (%) | CEA (%) | CD44 (%) | Cal (%) | HBME-1 (%) | Vim (%) | Des (%) | Thrombo (%) |
|-------------|-------------|-------|-----------|-------------|------------|---------|----------|--------|---------|--------|------------|---------|---------|-------------|
| Chhieng     | Cell blocks | SN    | -         | -           | -          | 84      | -        | 87     | -       | -      | -          | -       | -       | -           |
|             |             | SP    | -         | -           | -          | 88      | -        | 79     | -       | -      | -          | -       | -       | -           |
| Lozano      | Pap-stained | SN    | -         | 96.66       | 83.34      | -       | -        | -      | -       | -      | 100        | -       | -       | 100         |
|             | smears      | SP    | -         | 100         | 92.86      | -       | -        | -      | -       | -      | 80         | -       | -       | 76.66       |
| Politi      | Pap-stained | SN    | -         | 77.5        | 86.25      | -       | -        | -      | -       | -      | 100        | 98      | -       | -           |
|             | smears      | SP    | -         | -           | -          | -       | -        | -      | -       | -      | 80         | 71      | -       | -           |
| Murugan     | Cell blocks | SN    | -         | -           | -          | 100     | 97.44    | 92.31  | -       | -      | 100        | -       | 73.68    | 55.26       |
|             |             | SP    | -         | -           | -          | 97.37   | 68.42    | 76.32  | 92.31   | -      | -          | 79.49   | 95.12   | -           |
| Su          | Cell blocks | SN    | -         | 76.4        | 70         | 86.7    | 80       | 83     | -       | 79.2   | -          | -       | 47.2    | -           |
|             |             | SP    | -         | 86.8        | 92.5       | 98.1    | 96.2     | 88.3   | -       | 21.7   | -          | 70      | -       | -           |
| Barberis    | Destained   | SN    | -         | -           | -          | 72      | -        | -      | -       | -      | -          | -       | -       | -           |
|             | smears      | SP    | -         | -           | -          | 97      | -        | -      | -       | -      | -          | -       | -       | -           |
| Schofield   | Thin-prep   | SN    | -         | -           | -          | -       | -        | -      | 81      | -      | -          | -       | -       | -           |
|             |             | SP    | -         | -           | -          | -       | -        | -      | -       | -      | -          | -       | -       | -           |
| Morgan      | Cell blocks | SN    | -         | -           | 95         | -       | -        | -      | -       | -      | -          | -       | -       | -           |
|             |             | SP    | -         | -           | 100        | -       | -        | -      | -       | -      | -          | -       | -       | -           |
| Davidson    | Cell blocks | SN    | 79%       | 78          | -         | -       | 26       | -      | -       | -      | -          | -       | -       | -           |
| Current study | Cytospin   | SN    | -         | 98          | -         | -       | -        | 90     | -       | -      | -          | -       | -       | -           |
|             |             | SP    | -         | 86          | -         | -       | -        | -      | -       | -      | 100        | -       | -       | -           |

Ref.: reference number; SN: sensitivity; SP: specificity; EMA: epithelial membrane antigen; E-cad: E-cadherin; CEA: carcinoembryonic antigen; Cal: calretinin; Vim: vimentin; Des: desmin; Thrombo: thrombomodulin; Pap: Papanicolaou
for mesothelial (calretinin, EMA and HMGF-1) and adenocarcinoma [Ber-EP4, B72.3 and carcinoembryonic antigen (CEA)] on cell block preparations of effusion fluid samples. Although they found calretinin to be very sensitive and specific, they suggested addition of another antibody to improve results. Of the adenocarcinoma markers, their study showed Ber-EP4 to be the most sensitive followed by CEA. All the three adenocarcinoma markers were 100% specific. In the present study also, there was one case of malignant effusion and five of RMCH, which failed to show any reaction with either of the two antibodies. In addition, false-positive results were obtained with Ber-EP4 in seven cases of RMCH. Thus, there may be false-positive as well as false-negative reactions. Murugan et al. and several others have recommended the use of more than two antibodies. But a panel inclusive of only two antibodies will definitely be more cost-effective and will aid in making a correct diagnosis in most of the cases. Additional antibodies may be used where the results with these two antibodies are not conclusive enough. Other authors also have suggested two-antibody panels, though distinct from ours. We recommend the combination of the mesothelial marker, calretinin, and the epithelial marker, Ber-EP4, as a "primary" antibody panel, useful for distinguishing RMC from cells shed from carcinoma cells because of their associated high sensitivity and high specificity.

Most of the studies till date have been on sections cut from cell blocks [Table 3]. In their review on immunocytochemistry in effusion cytology, Fetsch et al. describe cell blocks to be the best preparation for performing ICC. Easy retrieval of archival material, similarity with surgical pathology material, and lack of much background staining are the reasons for their preference. Shidham and Atkinson have described the "subtractive coordinate immunoreactivity pattern" (SCIP) approach to localize, identify and characterize "second-foreign" population of cells in cell block sections by ICC. They recommend use of an immunopanel which will label the mesothelial and inflammatory cells. "Subtracting" the immunoprofiles of these components will highlight the unstained "second-foreign" population of cells, if present in the effusion sample. Additional antibodies may then be used to characterize these cells. This approach, however, is not feasible on cytology smears as it will not be possible to have the same cells and that too preferably in similar location, on different cytology smears while constructing coordinate immunoreactivity pattern for the various immunomarkers used. Thus, it can be used only on serial sections of cell blocks. Additionally, unlike cell block sections, it is not possible to assess multiple immunomarkers simultaneously on cytology smears, a technique useful to identify rare, singly dispersed malignant cells in effusion specimens, which may be easily missed in cytology smears. The best of immunostaining on cytology smears may not equate with immunopositivity/immunonegativity seen on formalin-fixed, paraffin-embedded tissue sections which form the yardstick to compare results for final interpretation of any immunoprofile. Thus, cytology smears may not be the ideal first choice for evaluating immunoprofile. Formalin-fixed cell block sections should be preferred over cytology smears for ICC.

In another study comparing results of ICC on ethanol-fixed smears, ethanol-fixed cell blocks and formalin-fixed cell blocks, Ueda and co-authors found the latter two to show significantly lower staining than ethanol-fixed smears. We evaluated ethanol-fixed cytospin smears and found ICC results comparable to those on cell block sections. There was minimal background staining which may have made interpretation of results difficult. Preparation of cytospin slides is an easy procedure, done routinely and does not require much additional effort in comparison to cell blocks. This may be used as an alternative to cell blocks at small centers with limited resources, where facilities for paraffin block preparation and cutting are not available.

**CONCLUSIONS**

Thus, a limited panel of calretinin and Ber-EP4 performed on cytospin slides is a cost-effective and time-saving technique, which may be used even for routine diagnostics in effusion cytology. It may be especially useful at resource-poor centers, especially those with a heavy workload, where these when used at the primary diagnostic level, may aid in rapid and accurate diagnosis in most morphologically difficult cases without much increase in the cost incurred.

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The authors declare that they have no competing interests.

**AUTHORSHIP STATEMENT BY ALL AUTHORS**

All authors of this article declare that we qualify for authorship as defined by ICMJE http://www.icmje.org/#author. Each author has participated sufficiently in the work and take public responsibility for appropriate portions of the content of this article. Each author acknowledges that this final version was read and approved.

**ETHICS STATEMENT BY ALL AUTHORS**

This study was conducted with approval from Institutional
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