Cytology Microarray on Cell Block Preparation: A Novel Diagnostic Approach in Fluid Cytology

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Abstract

Background: The cytological examination of serous body effusions to diagnose and stage malignancy is well accepted in clinical medicine. Conventional smear (CS) and cell block (CB) study has to be complemented with immunohistochemistry (IHC) for a definitive diagnosis of malignancy and also to differentiate it from reactive mesothelial cells. Cytology microarray (CMA) is a modification of tissue microarray which involves core needle biopsy of multiple cell blocks and embedding it in a single block. Aim: The aim of this study was to assess the effectiveness of IHC technique in CMA for rapid diagnosis of malignancy and to reduce the cost of testing. Materials and Methods: In this study, 82 pleural fluids were collected and subjected to CS and CB study followed by IHC in CMA blocks. Six commonly used antibodies were applied to confirm malignancy and diagnose the primary. Results: Nineteen cases were diagnosed as malignancy by CB method. MOC-31 confirmed adenocarcinoma deposit in 67% cases of which 44% were proved to be of lung primary by TTF1. Conclusions: IHC on CMA blocks of effusion fluids is a very effective technique that can significantly reduce the cost of testing by >70%.

Keywords: Cell block, conventional smear, cytology microarray

Introduction

The cytological study of different body fluids is a complete diagnostic modality in itself. It has increasingly gained acceptance in clinical medicine to such an extent that a positive diagnosis is often considered the definitive test and obviates explorative surgery. It is important not only in the diagnosis of malignant lesions, but also helps in staging and prognosis. Various methods are available like routine conventional smears (CS), cell blocks (CB) and ThinPreps from centrifuged deposit of fluid for cytological diagnosis. Yet the accurate identification of cells as either malignant or reactive mesothelial cells remains a genuine problem. Even if malignancy is identified in fluid, the exact origin of the cells is also difficult to ascertain in case of unknown primary. Hence for the definitive diagnosis immunohistochemistry (IHC) has to be applied in every single case which again increases the cost of test manifolds. We have herein used IHC in cytology microarray (CMA), a recent innovation in the field of pathology, to address the above-stated problem. Although tissue microarrays (TMA) have been widely used in the cancer research field for high-throughput gene expression analysis study and validation of tumour markers on tissue, CMA is still an unexplored area. There are only a few recent reports wherein cytology material has been used to construct microarray with success. The objective of our present study is to apply IHC technique in CMA prepared from CB of pleural fluids to reduce the expenditure of fluid cytology testing for confirmation of malignancy and searching for an unknown primary.

Materials and Methods

This study was conducted over a period of one and a half years. Average one or two pleural fluids were received daily. Detail history was taken from the patients retrospectively and all clinical findings along with reports were collected.

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very same day, smears were prepared from the centrifuged deposits and stained with the May-Grünwald Giemsa stain and Papanicolaou stain. Then a provisional diagnosis was given on the basis of the cytology study. The rest of the fluid from each sample was subjected to CB study by plasma thrombin method. Briefly, after centrifugation the supernatant was discarded and cell button was fixed overnight with five times volume of 10% formalin. On the next day it was washed with normal saline or phosphate buffer three times. Then equal volume of plasma and thrombin (2–3 drops) were added in the cell button and a viscid jelly like material was formed within 1–2 min. It was then scraped out and wrapped in filter paper and processed for routine histopathology. After 2 days we reported the fluid from CB section.

At the end of the week we collected all the donor blocks. Our experienced pathologists examined the slides to mark the area of interest with respect to maximum cellularity. A bone marrow biopsy needle (Jamshidi 11G) was used to obtain cell cylinders from the selected area of each CB. Punch-extracted cell cylinders were carefully transferred with forceps and arrayed on the recipient paraffin block box. One recipient box could accommodate 8–10 cylinders. Four micron paraffin sections were cut from the CMA blocks and deparaffinised through alcohols and xylene before immunostaining. A complete panel of IHC consisting of pankeratin, carcinoembryonic antigen (CEA), calretinin, leucocyte common antigen (LCA), MOC-31 and TTF1 were then applied to the malignant tumours for their exact categorisation.

**RESULTS**

Eighty-two pleural fluid samples were received in our study period. Maximum (21.95%) samples were collected from 50–59 years of age group. Males outnumbered females. Out of 82 patients, 27 (33%) were smokers and 9 (11%) had a history of tuberculosis. Bilateral effusion was present in 34.14% of cases.

Of the 82 cases, 62 were diagnosed as benign, 7 as suspicious and 13 as malignant by the CS. Most of the benign effusions were due to non-specific inflammation or reactive effusion. Twelve cases were of tubercular effusion (diagnosed by history, clinical examination, adenosine deaminase values and sputum tests) and one single case was of filarial effusion.

On the other hand in the CB study, 63 cases were diagnosed as benign and 19 as malignant. There was no such suspicious category in CB method. All seven samples that were reported as suspicious by the CS method were stamped as malignant by CB method. One case that was diagnosed as malignant in CS was actually florid proliferation of reactive mesothelial cells [Table 1].

Among the 19 malignant cases, the primaries were known in 7 cases which included 2 cases of carcinoma breast from female patients, 4 cases of carcinoma lung (3 male and 1 female) and 1 case of lymphoma. In the remaining 12 cases primary origins were not identified.

We have taken CB as gold standard as it is a modified histopathology method and CS is compared with respect to it. In our study, sensitivity for malignancy by CS method is 63% and specificity is 98%. Diagnostic yield for malignancy is increased by 9% if CB method is applied.

IHC was done on the microarray prepared from the CB of the 19 cases that were stamped as malignant by CB method. Although the haematoxylin and eosin staining of the CMA blocks were satisfactory [Figure 1], one sample fell off partially during the IHC heating procedure. As our primary aim was to confirm malignant effusion and differentiate metastatic adenocarcinoma from reactive mesothelial cells and mesothelioma, we have used a limited panel of IHC consisting of pancytokeratin, calretinin, CEA, MOC-31, TTF1 and LCA [Table 2]. Pancytokeratin was strongly positive in 16 out of the 18 cases (89%), moderately positive in 1 and negative in 1 case. That negative case was provisionally diagnosed as lymphoma in CB and was also positive for LCA. CEA was

| Table 1: Pleural fluid analysis (n=82) |
|---------------------------------------|
| Feature                      | Conventional smear method | Cell block method |
| Benign                      | 62                       | 63               |
| Suspicious                  | 07                       | 00               |
| Malignant                   | 13                       | 19               |
| Total                       | 82                       | 82               |

| Table 2: Immunohistochemistry of pleural fluid cell block microarray (n=18) |
|-------------------------------------------------|
| Antibodies                  | Positive (%) | Negative (%) |
| Pancytokeratin              | 17 (94%)     | 1 (6%)        |
| Leucocyte common antigen     | 1 (6%)       | 17 (94%)      |
| Calretinin                  | 0 (0%)       | 18 (100%)     |
| CEA                         | 9 (50%)      | 9 (50%)       |
| MOC-31                      | 12 (67%)     | 6 (33%)       |
| TTF1                        | 8 (44%)      | 10 (56%)      |

**Figure 1:** Representative photomicrograph of haematoxylin-eosin stained cell block cores assembled on cytology microarray. (Scanner view)
positive in nine cases (50%). Calretinin was uniformly negative in all 18 cases. MOC-31 strong membranous positivity was seen in 12 (67%) and strong nuclear positivity of TTF1 was noted in 8 of them (44%) [Figure 2].

**DISCUSSION**

The development of malignant pleural effusion (MPE) is a common complication of cancers of lung, breast and stomach.[7] Examination of fluids from the serous cavities of the body is thus an essential component of management in adult patients with suspected metastatic disease. CS examination, although a simple procedure done routinely, has a lot of limitations. Lack of tissue architecture, presence of reactive mesothelial cells, abundance of inflammatory cells and paucity of representative cells contribute to considerable difficulties in making conclusive diagnosis on CS.[8] A study by Oyafuso et al. on 4297 fluid samples showed the sensitivity, specificity, efficiency as well as positive and negative predictive values of smears as 44.55%, 95.7%, 50.1%, 98.7% and 20%, respectively.[9] Similar results were obtained by Motherby et al.[10] The unsatisfactory outcome of CS examination alone was evident from these two large cohort studies.

During reporting of pleural fluid, the problem which troubles us mostly is the similarity of atypical mesothelial cells with adenocarcinoma. The reactive mesothelial cells are usually common in pleurisy, pulmonary infarction and in cases of long-standing effusion. In that scenario mesothelial cells show reactive changes such as nucleomegaly, multinucleation, mitotic figures and high nucleocytoplasmic ratio. This problem may be compounded by the artefacts which are caused by poor fixation or staining technique.[6,11] These limitations of CS demanded the use of an adjuvant more confirmatory method for body effusion cell study. The CB technique as invented by Bahrenburg nearly a century ago came to the rescue.[12] It takes an intermediate position between histological and cytological techniques. Further, the effectiveness of the CB lies in the availability of diagnostic material for further histological examination, histochemistry and IHC studies. This helps in the better classification of the tumour and also identifies infectious causes by using microbiologic stains.[1,11,13,14]

In this study, we have made an attempt to prepare and analyse both CS and CB from the same sample. Special attention was given on age, sex, clinical, biochemical and radiological findings to arrive at final diagnosis.

The serial sections from paraffin blocks gave a concentrated material in smaller fields with a good estimation of the architectural pattern and cellular morphology. MPEs are often observed in lung cancer, especially adenocarcinoma, because it is a tumour that grows in the periphery of the lung and easily invades the pleural cavity.[15] In total, about 15% of patients have pleural effusion at the initial diagnosis of lung cancer. In this era of targeted therapy it becomes desirable to identify adenocarcinoma of lung from fluid and test the cancer cells for epidermal growth factor receptor (EGFR) gene mutation status that may aid in predicting the response of EGFR-tyrosine kinase inhibitor (TKI) gefitinib therapy.[16,17] EGFR TKIs have a higher response in specific subgroups of adenocarcinomas including females, never smokers and east Asians.[17,18]

In the current study, of the seven cases that were put under suspicious category by the CS method, six proved to be malignant and one benign by the CB method. The accuracy of the CB method was reinforced by IHC that also categorised each malignant tumour. According to various studies, an additional diagnostic yield for malignancy was noted if the CS technique was supplemented by the CB method.[13,19]

Here, in this study we have implemented the IHC method on CMA which is a modified version of TMA to reduce the cost of testing. TMA is a recently implemented, high-throughput technology used mostly in the analysis of molecular markers in oncology. The earliest reports of application of TMA was in 1998 from the National Human Genome Research Institute, Bethesda USA.[20,21] They worked on many different tumour types using various techniques and different markers. TMA allows more rapid validation of multiple immunohistochemical markers than the conventional individual case-based method.[22] It involves core needle biopsy of multiple pre-existing paraffin-embedded tissue blocks and re-embedding them in the form of an arrayed master block. Thus it means biopsy of a biopsy.[22] However it suffered from limitations related to sampling and sectioning of tissues. CMA was then developed as a quick, reproducible novel technique that was applicable to a wide range of cell suspension materials for genetic and immunomarker validation.[6,23]

The immunomarkers currently available for the recognition of adenocarcinoma in effusion include antibodies such as CEA, B72.3, Ber-Ep4 and MOC-31 and those available for the recognition of mesothelial cells include antibodies such as
calretinin, D2-40, CK5/6, WT-1 and mesothelin. MOC-31 has gained recognition in recent years as a highly reliable marker for distinguishing between metastatic adenocarcinoma and reactive or malignant mesothelial cells. We used a panel of six antibodies. Pankeratin confirmed the epithelial origin of the cells. The one pankeratin negative case was LCA positive. Calretinin was uniformly negative in all the 18 cases thus confirming that none of them were either mesothelioma or reactive mesothelial cells. Of the keratin positive ones, 50% were CEA positive and 67% MOC-31 strongly membranous positive. They were hence stamped as adenocarcinoma. TTF1 was then done to confirm lung primary. Eight of the cases were strongly positive for TTF1. The primary was known in four of them and the other four with unknown primaries were diagnosed as lung adenocarcinoma by TTF1. Among 17 pancytokeratin positive cases we could confirm adenocarcinoma in 12 cases by use of our panel of antibodies. However in five cases we could not provide the exact categorical opinion about their origin. This necessitates the scope of adding more antibodies to the panel to detect squamous, neuroendocrine or germ cell origin of metastatic deposits.

Though TMA has few disadvantages like reduction of the amount of tissue analysed and fear of antigenic changes in proteins by the fixation and embedding process, the problem can be overcome by accurately evaluating the original tumour tissues and also taking multiple cores from same sample. 

For identification of malignant effusion, differentiating it from mesothelial cells and for detecting unknown lung primary, we have used six common antibodies. Commercially a single antibody costs around 1000 rupees for each slide. The estimated cost of constructing the CMA for eight cases and staining six IHC slides is <10,000 rupees which translates into approximately 1500 rupees for each case. Whereas the cost of testing all six antibodies on individual CB sections would approximate to total 48,000 rupees that is 6000 rupees for each case. This results into huge savings that is particularly helpful in a country like ours with a population having financial crunch.

Thus to conclude, CB study is used in combination with smear study to diagnose malignancy in body fluids. The diagnostic yield increases manifolds if it is combined with IHC. This is particularly helpful in diagnosing tumours of unknown primary such as lung adenocarcinoma which has a specific-targeted therapy. The cost of IHC can be reduced considerably if it is done on microarray prepared from CB. In this study we have shown how with the help of few immunomarkers used in CMA we can confirm as well as categorise malignant cells in body fluid.

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Conflicts of interest
There are no conflicts of interest.

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