ANTICANCER RESEARCH *43*: 669-673 (2023)
doi:10.21873/anticanres.16204

Refined Diagnosis of Pleural Effusions by Immunocytochemistry of Cell Blocks

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Abstract. *Background/Aim: The main objective of microscopic examination of pleural effusions is to ascertain the presence of malignant cells. Effusions prepared routinely using May–Grünwald–Giemsa (MGG)- and Papanicolaou (PAP)-staining can, in a number of cases, provide inconclusive cytological results regarding malignancy. Patients and Methods: This report describes the refined diagnosis of such cases based on immunocytochemical analysis of pleural effusion cell blocks. Of the 340 pleural effusions obtained during 2019 at the Department of Clinical Cytology, Gävle Hospital, Sweden, 63 (18.5%) contained atypical cells of undetermined significance or potentially malignant cells. Results: This diagnosis could be refined using Epithelial Cell Adhesion Molecule/EPCAM (BEREP4) immunocytochemical analysis of effusion cell blocks, allowing previously inconclusive effusions to be classified as clearly benign 42/63 (66.7%) or malignant 21/63 (33.3%). Effusions initially diagnosed as clearly malignant (27/340; 7.9%) were all 27 (100%) BEREP4-immuno-stained. Most BEREP4-positive effusions (37/48; 77.1%) were also carcinoembryonic antigen (CEA) positive. The number of BEREP4-positive cells, however, tended to exceed that of CEA-positive cells. The BEREP4 positive effusions were further examined using different monoclonal antibodies, such as Thyroid transcription factor 1 (TTF-1) for primary pulmonary adenocarcinoma, to determine the original site of the primary tumour. Conclusion: Immunohistochemical staining of pleural effusion cell blocks significantly refines the diagnosis of serous pleural effusions, especially in cases where the preliminary diagnosis was atypical cells of undetermined significance or potentially malignant cells. Furthermore, in the cases of malignancy, the origin of the primary tumour could most often be determined.*

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Key Words: Pleural effusions, cell block, immunohistochemistry, malignant cells, BEREP4, CEA.

Serous effusions in the pleural cavity occur for three main reasons, namely: 1) cardiac and circulatory disturbances; 2) inflammatory or infectious diseases, and 3) metastatic spread of cancer, primarily adenocarcinoma (1, 2). Circulatory disturbances often result in bilateral pleural effusions, whereas unilateral effusions are more frequent in malignant diseases. The main objective of investigating serous pleural effusions by any Department of Pathology is to identify or exclude the presence of malignant disease (1, 2).

In Sweden, pleural serous effusions that are clearly benign or non-representative tend to be examined and diagnosed solely by cyto-technicians, whereas effusions containing atypical cells of unknown significance, potentially and/or clearly malignant cells, are transferred to the Clinical Cytology department for final evaluation and diagnosis by a clinical pathologist. At the Department of Pathology in Gävle Hospital, Sweden, over 300 pleural effusions are examined annually, which is estimated to correspond to over 15,000 examinations in Sweden per year. It is evident that cytological examinations of pleural effusions represent an important, routine diagnostic tool in Clinical Cytology.

The aim of this investigation was to refine the diagnosis of serous pleural effusions using immunostaining of cell blocks, a method that can considerably increase the sensitivity and specificity of cytological examinations compared to ordinary microscopic examinations of cytological smears routinely stained with May–Grünwald–Giemsa (MGG) and Papanicolaou (PAP).

Table I. *Correlation between the preliminary diagnosis of serous pleural effusions determined by cyto-technicians after light microscopy of MGG- and PAP-stained smears, and the diagnosis made by clinical pathologists following BEREP4-antibody staining of cell blocks.*

	BEREP4 Immunohistochemistry		
Ordinary light microscopy	Unstained (Benign)	Stained (Malign)	Total
Atypical cells of undetermined significance	34	8	42 (46.7%)
Potentially malignant cells	8	13	21 (23.3%)
Certain malignant cells		0	27 (30.0%)
Total	42 (46.7%)	48 (53.3%)	90 (100%)

Patients and Methods

All pleural effusions diagnosed at the Department of Clinical Cytology and Pathology, County Hospital, Gävle, Sweden, during 2019 were included in this analysis. Since the methodology for the preparation, staining, and examination of pleural effusions used here corresponds to routine clinical practise, this analysis was considered to be a quality assurance study as opposed to a purely scientific investigation. Thus, it was not subjected to ethical considerations or acceptance.

A total of 340 pleural examinations were collected at the Department during 2019, 207 (60.9%) from males and 133 (39.1%) from females aged between 16□99 years (median age 75 years). Two or more effusions were obtained in 65 (19.1%) of these individuals.

Of the total 340 pleural effusions, 250 (73.5%) were considered benign (and a few not representative) and had been diagnosed solely by cyto-technicians. The remaining effusions (90; 26.5%) were transferred to a clinical pathologist in Clinical Cytology after preliminary diagnosis by cyto-technicians that classified them as: 1) atypical cells of undetermined significance (n=42); 2) potentially malignant cells (n=21); or, 3) clearly malignant cells (n=21). Test tubes containing these pleural effusions (≥20 ml) were centrifuged (3,000 rpm for 10 minutes). The supernatant was removed and the basal precipitate in the test tube was exposed to two drops of normal human plasma (Department of Transfusion Medicine, Gävle Hospital) and two drops of thrombin (EMD Millipor Corp., Billerica, MA, USA). The precipitate was then fixed in 10% neutral buffered formalin (Solveco Chemicals, Rosersberg, Sweden). The precipitate was embedded in paraffin (Sakura Finetek, Göteborg, Sweden), sectioned at 4 μm and collected on glass slides (Matsunami, Osaka, Japan). One slide per effusion precipitate was stained with haematoxylin-eosin (Histolab products AB, Askim, Sweden), and two glass slides were immune-stained with BEREP4 (Agilent, Santa Clara, CA, USA) and CEA (Roche, Basel, Switzerland) monoclonal antibodies, respectively, in a Ventana instrument (Roche) according to a protocol described by Roche to identify malignant (3, 4) and/or adenocarcinoma (5) cells in the cell block, respectively. A cover glass (Tissue tek, Sakura Finetek) was placed on each stained section. Extra slides were prepared for potential analysis with additional immuno-stains.

In cases where malignant cells had been identified, additional antibodies were employed to identify the location of the primary malignancy, such as TTF-1 antibodies (Roche) for lung carcinoma (6); synaptophysin antibodies (Roche) for small cell lung carcinoma (SCLC); Caudal Type Homeobox 2 CDX-2 (Roche) and Cytokeratin 20 (Roche) antibodies for intestinal carcinoma; and, GATA-3 and oestrogen-receptor (ER), and Wilms tumour protein 1 (WT1) and PAX8 antibodies for breast and ovarian carcinoma, respectively (7-11). Immuno-stained slides were placed in a scanning microscope (Hamamatsu Nano Zoomer, S260 ×400) to obtain digital images of the cell blocks, which were examined and diagnosed by a clinical pathologist in Clinical Cytology on a data screen.

Results

A total of 90 pleural effusions examined by a clinical pathologist for diagnosis originated from 45 (50%) females and 45 (50%) males. The results following immunohistochemical analysis of the cell blocks using BEREP4 are shown in Table I and Figure 1. In all 27 effusions confirmed to contain malignant cells, BEREP4-immunoreactive cells were observed, corresponding to 100%. Pleural effusions with atypical cells of undetermined significance were BEREP4-positive in 19.0% (8/42) of cases and effusions containing potentially malignant cells were BEREP4-positive in 58.9% (13/21) of cases. Thus, in all 63 (18.5%) cases of pleural effusions containing atypical cells of undetermined significance or potentially malignant cells, BEREP4 immunostaining was discriminatory, thereby allowing classification of these effusions as clearly benign or malignant.

Although the sex distribution of effusions examined with BEREP4 antibodies was equal (45/45), BEREP4-positive effusions were more common in females, 73.3% (33/45), compared to males, 33.3% (15/45) ($p<0.000$). One reason for this discrepancy may be that effusions are more commonly collected in males with diseases not related to malignancy and that pleural effusion is often the first symptom of women with ovarian malignancies. The BEREP4 immunostaining also confirmed the initial diagnosis of clearly malignant cells in an additional 21 (6.2%) of effusions. In total, 14.1% (48/340) of the effusions were found to contain malignant cells after a refined diagnosis by BEREP4 immunostaining of the cell blocks.

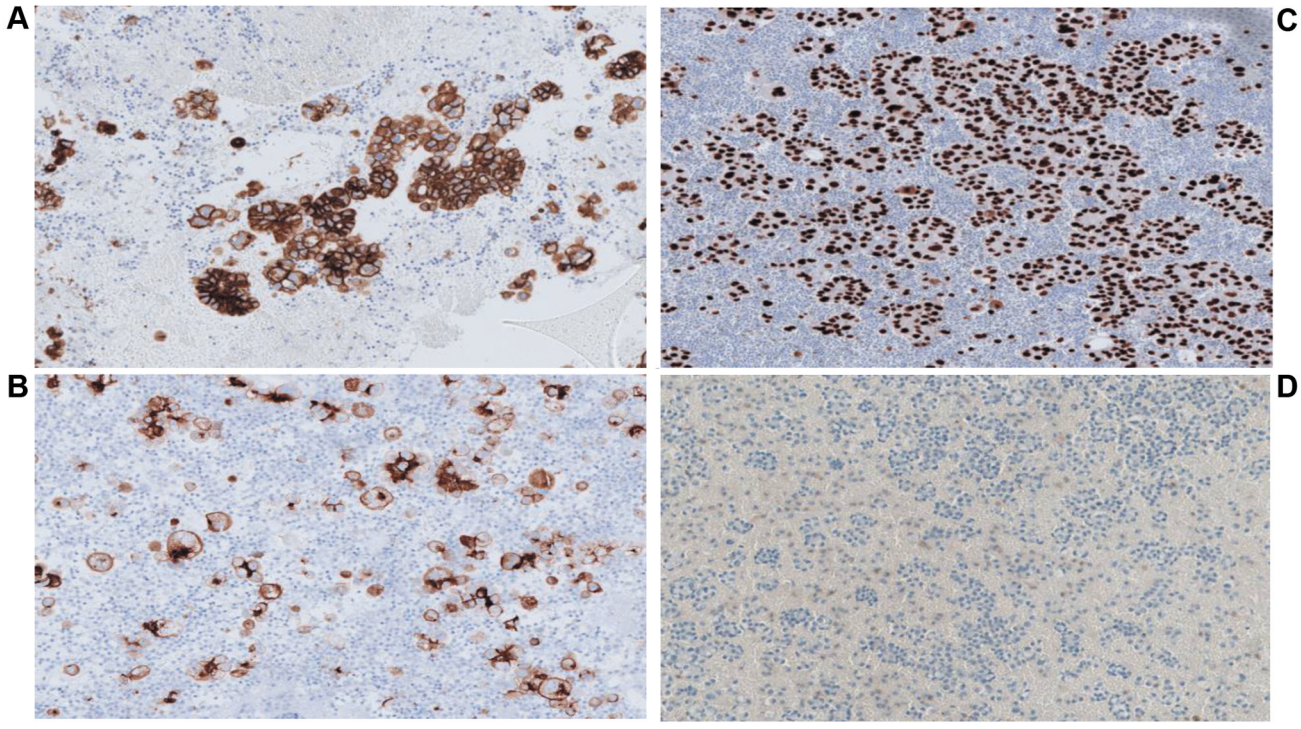

Figure 1. *Pleural effusion from a male with primary non-small cell carcinoma (NSCC). The tumour cells are (A) BEREP4-positive, (B) CEA- positive, and (C) TTF-1-positive, but cytokeratin 20-negative (D).*

Table II. *Correlation between carcinoembryonic antigen (CEA) immunoreactivity and sex in 48 BEREP4-immunoreactive cell blocks.*

	CEA Immunohistochemistry		
Sex	Unstained	Stained	Total
Females	8	26	34 (70.8%)
Males	3	11	14 (29.2%)
Total	11 (22.9%)	37 (77.1%)	48 (100%)

The BEREP4 staining was performed in conjunction with CEA staining in all 90 effusions considered as not clearly benign by the cyto-technicians. In BEREP4-positive cases, the correlation between CEA immunoreactivity and sex is shown in Table II. Most (77.1%; 37/48) BEREP4-positive effusions were also CEA positive; the number of BEREP4-positive cells, however, tended to exceed that of CEA-positive cells. This observation was mainly obvious in females with pleural metastases of ovarian carcinoma in which the CEA staining was weak or sometimes negative. The proportion of CEA-positive effusions did not show any obvious sex differences (76.4% and 78.6% in females and males, respectively). In most cases, the origin of the primary tumour site could be identified either by adding additional, topography-specific antibodies (*e.g.*, TTF-1 for lung adenocarcinoma; CDX-2 for lower intestinal adenocarcinoma; ER for gynaecological cancer; and synaptophysin for neuroendocrine tumours, such as SCLC) to the cell block or by analysis of corresponding patient medical records. Cell blocks were also used for quantitative analyses of programmed death-ligand 1 (PD-L1) immuno-reactivity in effusions containing primary lung adenocarcinoma cells, information which was of clinical importance and used to determine choice of therapy. Although cells from malignant lymphomas and mesotheliomas are also reported to occur in pleural effusions (12-16), this was not observed in the present study.

Discussion

In this investigation comprising a total of 340 pleural effusions, the use of BEREP4 immunohistochemistry on cell blocks of serous pleural effusions clearly enabled a refined diagnosis on benign/metastatic status compared to methodology based solely on routine MGG-stained smears. In 63 (18.5%) of cases with atypical cells of undetermined significance or potentially malignant cells, BEREP4 immunostaining was discriminatory in 100% of cases, and allowed the classification of these effusions as clearly benign or malignant, as reported previously (3, 4, 12, 13).

The BEREP4 antigen is a general marker for epithelial cells and malignant epithelial tumours, such as squamous carcinoma and adenocarcinoma, as shown in this study. The BEREP4 antibody has been used to discriminate mesotheliomas from metastatic cancer but can also be employed to identify malignant cells of epithelial origin in pleural effusions containing cells of undetermined significance or potentially malignant cells (3, 4, 12-14). In contrast, the CEA antigen is considered to be a general marker for the identification of adenocarcinoma cells (5). In this study, BEREP4-positive effusions were more common in females, 73.3% (33/45), compared to males, 33.3% (15/45). One reason for this discrepancy may be that effusions are more commonly collected in males with diseases not related to malignancy and that pleural effusion is often the first symptom of women with ovarian malignancies. However, some types of adenocarcinomas are either CEA-negative or infrequently immuno-stained, such as breast adenocarcinoma and ovarian adenocarcinoma. In this investigation, pleural effusions with malignant cells of unknown origin were shown to be metastases of ovarian carcinoma after application of ER, WT1, and PAX8 antibodies, and subsequently verified by the clinical examination (7, 10, 11).

Two primary goals exist for the examination of pleural effusions for diagnosis, namely: 1) to clarify whether malignant cells are present or not; and 2) to identify the primary, topographical tumour site when an effusion with malignant cells of unknown origin is observed. Often, the presence of a malignant tumour is already known, in which case the demonstration of malignant pleural effusions has clinical implications for choice of further anti-tumour treatment. Not infrequently, however, a pleural effusion with malignant cells is often the first indication of a malignant disease. For instance, the initial manifestation of a malignant condition is the occurrence of pleural metastases derived from a serous ovarian cancer. In such cases, using more specific antibodies (*e.g.*, ER, WT1 and PAX8) can provide conclusive information on the topographical site of the primary tumour.

Refined diagnosis of pleural effusions has further implications. Cell blocks containing malignant cells of defined types of carcinomas can be sent to molecular pathology laboratories for molecular determination of the different types of DNA abnormalities fundamental to determining relevant anti-tumour therapy. They can also be employed for more selective immuno-staining. Sections from such blocks can be stained with PD-L1 antibodies, which provide quantitative data based on comparing the number of BEREP4- versus PD-L1-positive cells in the immuno-stained sections. Such information may be of critical value when evaluating optimal therapeutic strategies in primary lung adenocarcinoma patients (17).

In conclusion, immunohistochemical staining of cell blocks with BEREP4 antibodies can significantly refine the diagnosis of pleural effusions, especially in cases where preliminary diagnosis of effusions with atypical cells is of undetermined significance or a potential malignancy. Moreover, by extending the examination using more site-specific antibodies, the origin of the primary tumour can be identified in most cases.

Conflicts of Interest

The Authors report no conflicts of interest in relation to this study.

Authors' Contributions

The Authors equally contributed to all aspects of this study.

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Received September 26, 2022
Revised December 15, 2022
Accepted December 20, 2022