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Detection of cancer cells in effusions from patients diagnosed with gynaecological malignancies

Evaluation of five epithelial markers

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Abstract The detection of malignant cells in pleural, peritoneal, and pericardial fluids of cancer patients marks the presence of metastatic disease and is associated with a grave prognosis. We evaluated five epithelial markers for the detection of cancer cells in 94 fresh pleural, peritoneal and pericardial effusions. Eighty-four of the samples were regarded as adequate for analysis after evaluation of cytological smears, including 61 samples from patients known to have gynaecological neoplasms. The other 23 samples were from patients with various non-gynaecological malignancies or tumours of unknown origin. Our control cases were 10 fallopian tubes not affected by any malignancy and 12 malignant mesotheliomas. Cell blocks from all cases were stained for CA-125, BerEP4, carcinoembryonic antigen (CEA), BG8 (Lewis Y blood antigen), and B72.3 (TAG-72). Fifty-one of 84 cases were diagnosed as malignant or suggestive of malignancy in cytological smears and/or cell block sections. However, staining for epithelial markers highlighted the presence of malignant cells in 7 additional cases. When membrane staining was evaluated, the sensitivity of the markers studied in detecting malignant cells was as follows: CA-125: 88%, BerEP4: 78%, CEA: 26%, BG8: 86%, B72.3: 79%. Membrane positivity for CEA, B72.3 and BerEP4 was not detected in reactive mesothelial cells. However, membranous staining in mesothelial cells was evident in 13% and 31% of cases with the use of BG8 and CA-125, respectively. Weak cytoplasmic staining for CEA was observed in mesothelial cells in 2 cases. When Ber-EP4, B72.3, and BG8 staining results in cancer cells were combined, the following sensitivity levels were observed: BG8+B72.3: 91%; BG8+Ber-EP4: 90%; B72.3+Ber-EP4: 93%; BG8+Ber-EP4+B72.3: 95%. The detection of malignant cells in effusions is facilitated by the use of immunocytochemistry using a wide panel of antibodies. BerEP4 and B72.3 appear to be the best markers when both sensitivity and specificity are considered, followed by BG8, while CEA and CA-125 have a limited role in the detection of metastases from gynaecological tumours owing to the low sensitivity of the former and the low specificity of the latter. Analysis of all staining results should be based on a thorough morphological examination.

Key words Gynaecological cancers · Effusions · Epithelial markers · Immunocytochemistry

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Introduction

Malignant neoplasms are characterized by their ability to metastasize. Tumour spread frequently involves the serosal surfaces, most often with ovarian and breast carcinomas in female patients [5]. However, the detection of malignant cells in routine cytological preparations from body cavity effusions can be extremely difficult. Hyperplastic mesothelial cells, seen in various benign clinical settings, undergo marked nuclear and cytoplasmic alterations, some of them mimicking the morphology of malignant cells [5, 6]. Furthermore, these changes may follow radiation or chemotherapy, which are common adjuncts to surgery in a wide variety of cancers [5].

In recent years, a large number of antibodies have been evaluated for their ability to differentiate adenocarcinomas from malignant mesotheliomas in tissue sections [7, 8, 30, 31]. Other studies have attempted to differentiate carcinoma cells from benign reactive and hyperplastic mesothelial cells in effusions [11, 14, 26, 33, 35]. Variable results regarding both sensitivity and specificity were recorded in these studies, in part because car-

cinomas with different origins were included. Limited data are available on the optimal immunohistochemical panel for the detection of cancer cells originating from gynaecological malignancies in effusions.

The present study evaluated the staining pattern of benign mesothelial cells and malignant cells in pleural, peritoneal, and pericardial effusions of gynaecological cancer patients by immunocytochemistry.

Materials and methods

The material studied was 94 consecutive fresh nonfixed peritoneal, pleural, and pericardial effusions submitted to the Section of Cytopathology, Department of Pathology, The Norwegian Radium Hospital, during the period of January to August 1998. All specimens were obtained from patients with either a previous diagnosis of cancer or a clinical suspicion of malignancy. Patient ages ranged from 16 to 86 years. Eighty-four of the samples were regarded as adequate for analysis after evaluation of sample size and cell preservation. These included 61 samples from patients with known gynaecological neoplasms. 18 cases various of non-gynaecological malignancies and 5 cases with unknown primary tumours were also studied. Control specimens were 10 nonmalignant fallopian tubes and 12 malignant mesotheliomas, all from formalin-fixed paraffin-embedded histology sections. The distribution of cases according to tumour origin and histological diagnosis is shown in Table 1.

All specimens were received as fresh nonfixed effusions, volume range 20–2000 ml. The specimens were centrifuged in an Eppendorf 5810 centrifuge (Hamburg, Germany) for 10 min at 2000 RPM. Each resulting pellet was used for the preparation of four cytological smears (2 alcohol-fixed Papanicolau-stained, and 2 air-dried Diff-Quik-stained smears) and a formalin-fixed, paraffin-embedded cell block. The remaining material was frozen in RPMI+DMSO medium for future studies. Cell blocks were prepared using the Shandon Lipshaw cytoblock kit (Shandon, Pittsburgh, Pa.).

Cytological smears from all samples were evaluated by a senior cytopathologist and reported as positive, suggestive or negative for malignant cells. Owing to the possibility of sampling a different population in cell blocks, sections from all cell blocks were evaluated in a double–blind manner and graded as described above by two senior cytopathologists (A.B. and B.R.). The diagnostic criteria used were those in the guidelines published by Bedrossian [5]. All cases were then reviewed by three of the authors (A.B., B.R. and B.D.) before evaluation of the immunocytochemistry results. For cases in which discrepant morphological diagnoses were obtained, a discussion was held, in which the slides were re-evaluated until a consensus diagnosis was achieved.

For immunocytochemistry, formalin- fixed paraffin-embedded sections, 4 µm thick were mounted on silane-coated slides. After air drying at 37°C for 24 h, slides were deparaffinized and rehydrated. Staining was performed with labelled Avidin–Biotin (LAB) [15, 16]. Negative control sections were subjected to a similar staining procedure, with exclusion of the primary antibody application. The primary antibodies and antigen retrieval protocols, where used, are listed in Table 2. Four breast carcinoma cell lines (T-47D, MCF7, SK-BR-3, and MDA-MB-231) were tested as positive controls for all five antibodies, using cell blocks that were prepared using the procedure detailed above. One consistently positive cell line was chosen for each antibody and used in every staining reaction, as detailed in Table 2.

The presence of staining in malignant/suggestive cells and in benign mesothelial cells was evaluated by the authors after evaluation of the morphological findings. Staining intensity was scored semi-quantitatively as 0, 1, 2, 3, corresponding to absent, weak, moderate or strong staining. In addition, a minimum of 200 cells (if available) were evaluated to determine the percentage of stained cells. All morphology-negative, immunocytochemistry-positive cases were re-evaluated morphologically to verify the existence of a malignant cell population.

Table 1 Case distribution according to tumour origin and histological diagnosis

Diagnosis	No. of cases
Ovary:adenocarcinoma	40
Ovary: borderline tumour	2
Ovary: benign cyst	2
Uterus: Endometrial adenocarcinoma	11[a]
Uterus: carcinosarcoma	1
Uterus: leiomyosarcoma	1
Cervix: adenocarcinoma	2
Cervix: squamous cell carcinoma	1
Vulva: squamous cell carcinoma	1
Primary peritoneal carcinoma	3
Breast: adenocarcinoma	11
Lung: adenocarcinoma	1
Colon: adenocarcinoma	1
Osteosarcoma	1
Malignant lymphoma	1
Unknown origin	5
Control: malignant mesothelioma	12
Control: benign uterine tubes	10
Total	106

[a] One patient had a concomitant breast adenocarcinoma

Results

Forty-five of 84 cases (54%) were classified as malignant; 1 case (1%) was interpreted as suggestive of malignancy; and 38 (45%) were classed as benign in the morphological evaluation of cytological smears. The combined morphological evaluation of smears and cell block sections resulted in the diagnosis of malignant cells in 5 more specimens [total=51 (=61%) positive/suggestive specimens]. Thirteen specimens contained malignant cells, either exclusively or with an accompanying inflammatory infiltrate, without a significant mesothelial cell population. Immunocytochemistry staining results

Table 2 Antibodies used in the study

Antibody	Source	Dilution	Antigen retrievel	Control cell line
CA-125	Dako (Glostrup, Denmark)	1:50	Microwave 2×5 min, citrate	SK-BR-3
CEA (monoclonal)	Dako	1:50	Trypsin	T-47D
BerEP4	Dako	1:50	None	MCF7
BG8	Signet (Dedham, Mass)	1:50	Microwave 2×5 min, citrate	T47-D
B72.3	Biogenex (San Ramon, Calif.)	1:50	Microwave 2×5 min, citrate	MDA-MB-231

Table 3 Immunocytochemistry staining results [a]

Class		Intensity				Total positive
		0	1	2	3	
Malignant/atypical epithelial cells	CA-125	7	0	5	46	51/58 (88%)
	CEA	38	1	7	12	20/58 (34%)
	Ber-EP4	13	0	11	34	45/58 (78%)
	BG8	8	0	9	41	50/58 (86%)
	B72.3	12	0	1	45	46/58 (79%)
Benign mesothelial cells	CA-125	49	0	1	21	22/71 (31%)
	CEA	69	2	0	0	2/71 (3%)[b]
	Ber-EP4	71	0	0	0	0/71 (0%)
	BG8	62	1	2	6	9/71 (13%)[c]
	B72.3	71	0	0	0	0/71 (0%)
Malignant mesotheliomas	CA-125	4	0	0	8	8/12 (67%)
	CEA	12	0	0	0	0/12 (0%)
	Ber-EP4	12	0	0	0	0/12 (0%)
	BG8	12	0	0	0	0/12 (0%) [c]
	B72.3	12	0	0	0	0/12 (0%)
Fallopian tubes	CA-125	0	0	0	10	10/10 (100%)
	CEA(cytoplasm)	4	2	4	0	6/10 (60%)
	Ber-EP4 (cytoplasm)	2	0	4	4	8/10 (80%)
	BG8	1	0	4	5	9/10 (90%)
	B72.3	8	0	2	0	2/10 (20%)

[a] Refers to membranous staining using all antibodies, with exclusion of CEA, for which the combined value of cytoplasmic and/or membranous staining in malignant cells is given
[b] Cytoplasmic staining
[c] Isolated cytoplasmic positivity for BG8 was observed in benign mesothelial cells in a further 6 cases, and in 1 malignant mesothelioma

Table 4 Staining results in 7 malignant effusions associated with benign smears and cell block diagnoses

Case no.	Origin	CA-125	CEA	BerEP4	BG8	B72.3
1	Ovary	+	+	–[a]	+	+
2	Ovary	+	–	–[a]	–	–
3	Ovary	+	–	–	+	+
4	Cervix	–	–	+	+	+
5	Endometrium	–	–	–	+	+
6	Breast	–	–	–	+	+
7	Breast	–	–	+	+	–

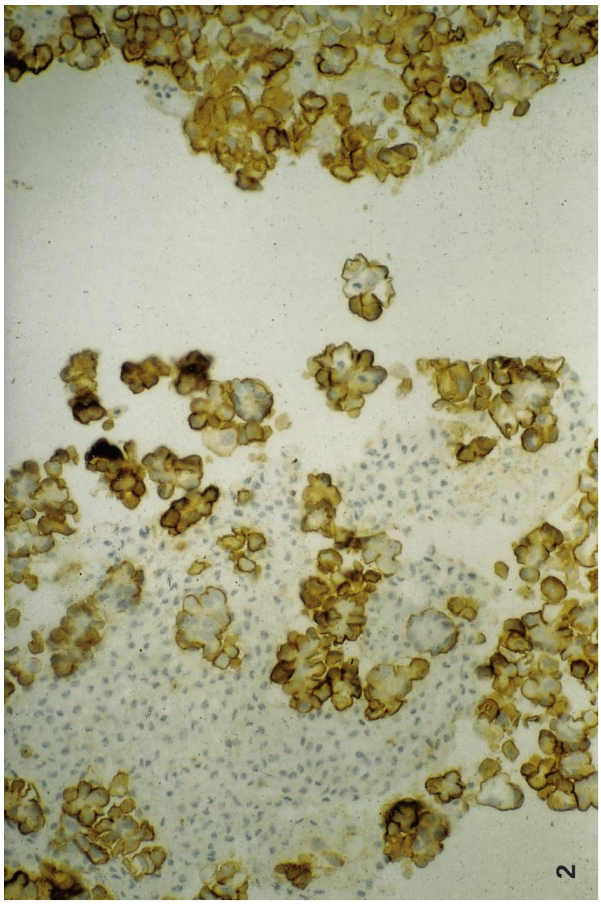
[a] Intense cytoplasmic positivity observed

for malignant/suggestive cells, benign mesothelial cells, and the control groups are detailed in Table 3. Immunocytochemically positive atypical or clearly malignant cells were detected in all 51 cases in which the smears and/or cell blocks morphologically positive, and also in 7 cases that were interpreted as benign in cytological evaluation of both smear and cell block morphology (total = 58 cases) (Fig. 1–4). All positive cases showed staining for two or more markers. The morphological and immunocytochemical results for the discrepant cases are shown in Table 4. The immunocytochemical profiles of carcinomas of gynaecological and breast origin differ only with CA-125 immunostaining (Table 5). When membrane staining was evaluated, the sensitivity of the markers in detection of malignant cells in the above 58 cases was as follows: CA-125: 88%, BerEP4: 78%, CEA: 26%, BG8: 86%, B72.3: 79%. Inclusion of cases with cytoplasmic positivity for CEA increased its sensitivity to 34%. Membrane positivity for CEA, B72.3 and BerEP4 was not detected in reactive mesothelial cells, although isolated cytoplasmic positivity for CEA and BerEP4 was seen in 2 and 5 cases, respectively. In contrast, it was evident in 13% and 31% of cases with the use of BG8 and CA-125, respectively. When Ber-EP4,

Table 5 Staining results according to tumour origin in tumour-positive cases [a]

Origin	CA-125	CEA	BerEP4	BG8	B72.3
Ovarian carcinoma	32/33 (97%)	11/33 (33%)	26/33 (79%)	29/33 (88%)	28/33 (85%)
Endometrial carcinoma [b]	5/5 (100%)	3/5 (60%)	4/5 (80%)	5/5 (100%)	5/5 (100%)
Cervical carcinoma	1/2 (50%)	0/2 (0%)	2/2 (100%)	1/2 (50%)	2/2 (100%)
Breast carcinoma	6/10 (60%)	4/10 (40%)	8/10 (80%)	10/10 (100%)	7/10 (70%)
Other tumours [c]	7/8 (87%)	2/8 (25%)	5/8 (62%)	5/8 (62%)	4/8 (50%)
Total	51/58 (88%)	20/58 (34%)	45/58 (78%)	50/58 (86%)	46/58 (79%)

[a] Refers to membranous staining for Ber-EP4, BG8, B72.3, and CA-125, and to cytoplasmic and/or membranous staining for CEA
[b] Including 1 carcinosarcoma
[c] Including two cases of primary peritoneal carcinoma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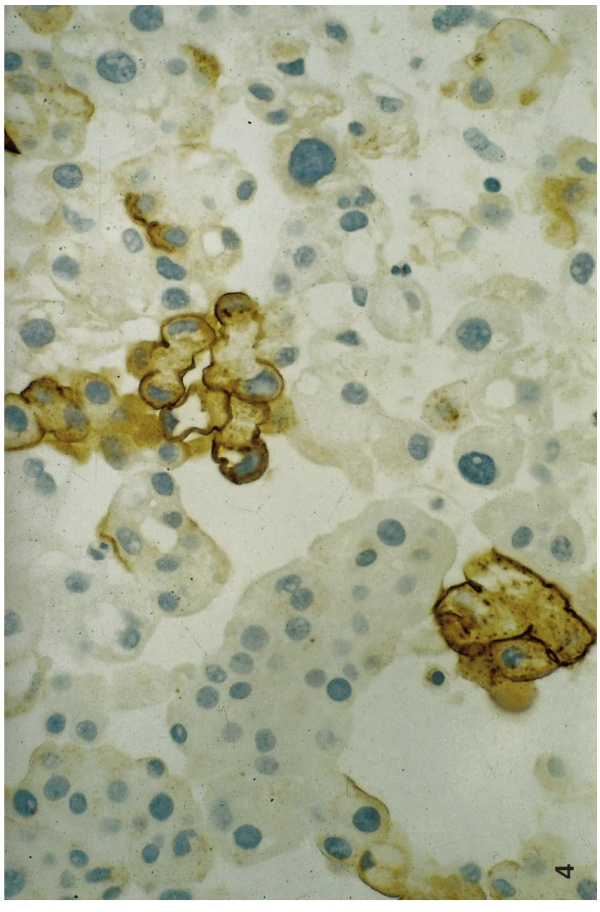
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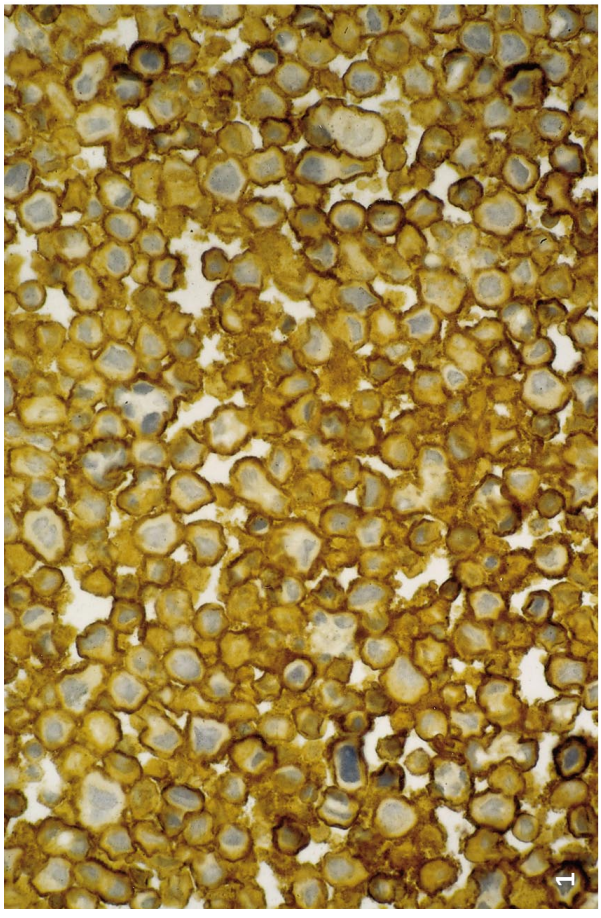
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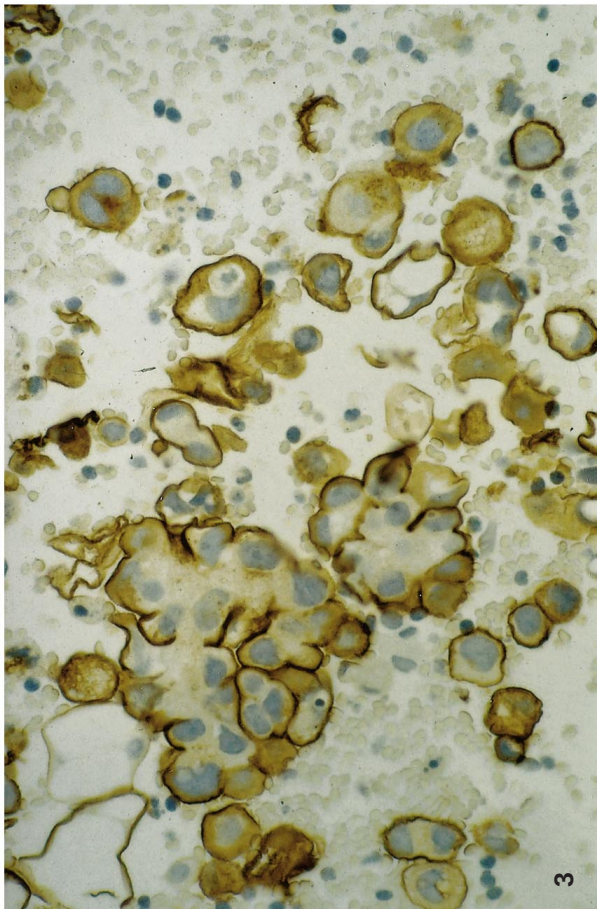
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B72.3, and BG8 staining results in cancer cells were combined, the following sensitivity levels were observed: BG8+B72.3: 91%; BG8+Ber-EP4: 90%; B72.3+Ber-EP4: 93%; BG8+Ber-EP4+B72.3: 95%. The number of cells stained varied with the antibody used. CA-125 immunostaining was diffuse in both malignant epithelial and reactive mesothelial cells, when present, being 100% in cancer cells in 25 specimens. BG8, B72.3 and BerEP4 stained tumour populations of variable size, most often between 25% and 75% of cells. CEA positivity was limited to only a few cells (<10%) in most positive cases. Mesothelial cell positivity to BerEP4, BG8, and CEA, when present, tended to be focal.

Discussion

In the present study, the role of five monoclonal antibodies in the detection of cancer cells in 84 effusions was evaluated.

Differentiation between metastatic adenocarcinomas and malignant mesotheliomas can at times be extremely challenging, and several studies have evaluated large antibody panels in attempt to establish the optimal immunohistochemical profile for the distinction between these two entities [7, 8, 30, 31]. Both conditions are associated with a poor prognosis, but malignant mesotheliomas are not often seen in routine surgical pathology or in diagnostic cytology. It is thus the distinction of adenocarcinoma cells from reactive mesothelial cells that most frequently leads to diagnostic difficulties. The tendency of ovarian carcinomas to shed cells in ascitic fluid, and the ability of proliferating reactive mesothelial cells to exhibit nuclear and cytoplasmic changes that mimic malignant cells complicate the matter further. Furthermore, the immunohistochemical profile of malignant mesotheliomas does not appear to be identical to that of benign mesothelial cells [11, 17].

The clinical role of CA-125 as a tumour marker in ovarian carcinomas is well established. However, since the initial report of Bast et al. [3], several studies have demonstrated CA-125 positivity in the benign epithelium of the female genital tract, and also in the normal lung, serous membranes, various inflammatory processes, and malignant mesotheliomas [4, 9, 19, 27, 28]. In contrast, no CA-125 positivity was found in adult ovaries [37], and weak to negative staining was seen in cultured mesothelial cells [36]. Most of the above studies have not used antigen demasking of any kind, and the reported pattern of staining in these studies is either cytoplasmic or membranous. We used microwave oven pretreatment with citrate buffer for CA-125 immunostaining. This resulted in intense specific membrane staining in all positive cases, often in 80–100% of tumour cells (Fig. 1). CA-125 positivity was detected in 90% of carcinomas in the present study, being seen most often in ovarian and endometrial tumours (97% and 100%, respectively). These results are in agreement with other studies of ovarian carcinomas [27, 37]; the moderate enhancement in sensitivity possibly resulted from our antigen exposure procedure. Notably, all fallopian tube controls were CA-125 positive, as described in the above studies. More significantly, mesotheliomas and benign mesothelial cells exhibited a similar staining pattern in a large number of cases. We believe that these findings preclude the use of CA-125 in the diagnostic work-up for effusions. Moreover, the high positivity rate in breast carcinomas may render the distinction between these two tumours impossible in an isolated case.

Carcinoembryonic antigen (CEA) has been evaluated as a marker for malignant epithelial cells in effusions in numerous studies [1, 2, 11, 14, 20, 22, 23, 26, 27, 32, 33, 35]. The sensitivity of CEA immunostaining in these studies ranges from zero to 100%. However, a comparative evaluation of these reports is impossible, as both polyclonal [14, 22, 26, 27] and monoclonal [11, 20, 33, 35] CEA was used, there is a lack of detail on the antibody specificity [1, 2, 32] and on the tissue origins of the malignant cells that were evaluated [2, 14, 32], and the tumours of origin vary, including others besides those of the breast or the female genital tract [23]. In four studies in which a monoclonal anti-CEA was applied, and in which results for ovarian carcinomas are specified, the reported sensitivity ranges from zero to 50% [11, 20, 33, 35]. Our results (sensitivity=34%) are in agreement with those of Delahaye et al. [11]. The specificity in these studies ranged from 94.7% to 100%, as it did in our hands (specificity=97% for cytoplasmic staining, 100% for membranous staining in benign mesothelial cells, specificity= 100% in mesotheliomas). Despite its high specificity, the low sensitivity of CEA in the detection of metastatic ovarian tumours argues against its inclusion in a limited panel for the detection of metastatic gynaecological malignancies, unless the original diagnosis (e.g. mucinous carcinoma) supports its use.

Since the first report on Ber-EP4 in 1989 [21], its role in the diagnosis of epithelial malignancies has been the subject of a large number of studies [2, 4, 11–13, 20, 24, 25, 29–32, 35] which have been reviewed by Ordonez [29]. The results vary widely, with a reported sensitivity of 32–100% and specificity of 12–100% [29]. Nevertheless, nine studies of effusion specimens have demonstrated a specificity of 98–100%, although with a widely discrepant sensitivity [2, 11–13, 20, 24, 25, 32, 33]. BerEP4 staining in carcinoma cells was observed in 88% of the cases in this study, in agreement with three of the above

Fig. 1 Intense membranous and weak cytoplasmic CA-125 staining of all malignant cells in a case of poorly differentiated ovarian carcinoma. ×100

Fig. 2 Diffuse membranous and focal cytoplasmic Ber-EP4-positivity in papillary ovarian adenocarcinoma cells. Adjacent mesothelial cells are negative. ×50

Fig. 3 Intense diffuse staining for BG8 in a cervical adenocarcinoma. ×100

Fig. 4 Focal B72.3 positivity in an ovarian papillary adenocarcinoma. ×100

studies [12, 13, 32]. As with the above-mentioned antibodies, no difference was observed between ovarian and breast carcinomas. All control mesotheliomas were negative. Isolated cytoplasmic positivity was observed in benign mesothelial cells in a few cases, as well as in some carcinomas. However, when membranous staining alone was evaluated no mesothelial positivity was observed, with an adjusted sensitivity of 78%. Moreover, the frequent positivity for Ber-EP4 in benign tubal cells supports its initially reported ability to stain various epithelial cells. Our findings support the inclusion of Ber-EP4 in any panel designed for the diagnosis of malignant effusions.

The role of ABH blood antigens in tumour metastasis and their association with survival in the case of solid tumours has been the subject of a number of studies, and Lewis^y, an AB precursor antigen, was postulated to have a role in tumour spread [10]. Interestingly, membranous staining for Lewis^y was observed in pulmonary adenocarcinomas, but not in malignant mesotheliomas, in a study by Jordon et al. [18]. BG8, an antibody against Lewis^y antigen, showed promising results in a study of adenocarcinomas and mesotheliomas in tissue specimens [31], in which the observed sensitivity and specificity were 88.6% and 91.3%, respectively. To the best of our knowledge, no studies of effusions have been published previously. In our study, in agreement with the above report, the sensitivity and specificity of BG8, when membranous staining was evaluated, were 86% and 87%, respectively. These results were substantiated by our control results, 90% of control fallopian tubes, but none of 12 mesotheliomas, showing membrane positivity. These findings support the inclusion of BG8 in our effusion panel, and bring forward the possibility of using other carbohydrate antigens for diagnostic purposes.

B72.3 is a tumour-associated glycoprotein and a widely used marker of epithelial malignancies [34]. Few antibodies have demonstrated such consistent results in the evaluation of specificity in both histology specimens and cytology samples, the range being 96–100% [2, 7, 8, 11, 14, 22, 26, 30, 31, 35, 37]. The reported sensitivity in these studies varied from 69% to 100%, with the notable exception of one study of ovarian tumours, in which only 33–50% of the tumours stained positive, depending on their histological subtype [37]. Our results are in agreement with those of the studies mentioned above, with sensitivity and specificity of 79% and 100%, respectively. Interestingly, clear membrane immunoreactivity was observed in all positive cases, often accompanied by a granular cytoplasmic pattern. The former was reported by Bollinger et al. [7], while an exclusively granular pattern was described in two other studies [30, 31].

The morphological diagnosis of malignant cells in effusion specimens can be an extremely difficult task. The reported rate of false-negative diagnosis was as high as 42% in a large series of 6000 patients [5]. In the present study, 7 specimens that were diagnosed as benign in both cytological smears and cell block sections proved to contain malignant epithelial cells that were highlighted by immunocytochemical staining. In 10 other cases, atypical or malignant cells were detected only with one of the two diagnostic techniques. The latter finding may result from interobserver differences. However, it may also be attributed to the paucity of tumour cells in some samples. These cases appear to benefit from the use of cell block diagnosis. Regardless of the underlying cause of false-negative morphological diagnosis, immmunocytochemistry aids in highlighting tumour cells that are underdiagnosed in a considerable number of cases.

No false-positive cases were seen in this study, in agreement with the much lower reported rate compared with false-negative diagnoses [5]. This may result from the caution that is accepted to be necessary in the diagnosis of effusions, which results in a tendency to diagnose as malignant only cases that have a large and unequivocal cancer cell population. Thus, it is cases that are characterized by a small malignant population, often masked by an inflammatory infiltrate or proliferating mesothelial cells, that would benefit most from the use of immunocytochemistry.

For patients diagnosed with gynaecological malignancies, the combined use of Ber-EP4, B72.3, and BG8 appears favourable, while CA-125 and CEA cannot be recommended for inclusion in a diagnostic panel. The combination of any two of these three antibodies (Ber-EP4, B72.3, and BG8) enhances the diagnostic yield, but the use of all three results in the highest sensitivity, leading to the diagnosis of any malignant cells in the vast majority of cases. Evaluation of additional markers (such as carbohydrate surface antigens) may make a further contribution to the correct diagnosis of such patients.

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