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Ber-EP4 and Anti-Calretinin Antibodies: A Useful Combination for Differential Diagnosis of Various Histological Types of Ovarian Cancer Cells and Mesothelial Cells

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Okamoto, S., Ito, K., Sasano, H., Moriya, T., Niikura, H., Terada, Y., Sato, S., Okamura, K. and Yaegashi, N. *Ber-EP4 and Anti-Calretinin Antibodies: A Useful Combination for Differential Diagnosis of Various Histological Types of Ovarian Cancer Cells and Mesothelial Cells*. Tohoku J. Exp. Med., 2005, **206** (1), 31-40 —— The differential diagnosis between reactive mesothelial cells and ovarian carcinoma cells is often difficult in cytologic specimens. Immunocytochemical procedures have been utilized in assisting this differential diagnosis, with limitations. Furthermore, previous studies examined only serous type but not other histological types of ovarian carcinoma cases. Therefore, we evaluated the practical value of various epithelial and mesothelial markers in differential diagnosis of these two types of cells. Various types of ovarian carcinoma (serous, $n = 22$; mucinous, $n = 10$; endometrioid, $n = 7$; clear cell, $n = 10$) and benign mesothelial tissues ($n = 15$) were studied by immunohistochemistry. We then studied effective panels of antibodies by immunohistochemistry in 43 cytologic specimens of ascites or peritoneal lavage fluid consisting of 20 reactive mesothelium and 23 adenocarcinomas of the ovary. In the tissue specimens, Ber-EP4, a monoclonal antibody of epithelial antigen, and a polyclonal antibody against calretinin, which is expressed in mesothelium, are used in differentiating reactive mesothelial cells from ovarian carcinoma. In cytologic specimens, the sensitivity and specificity of Ber-EP4 were 100% and 90%, respectively. The sensitivity and specificity of the anti-calretinin antibody were 90% and 91%, respectively. Using multiple regression analysis, the correlation coefficient between epithelial antigen and calretinin reactivity was $r = 0.938$, with a significance level of $p < 0.0001$. In conclusion, the combined immunostaining of cytologic specimens for Ber-EP4 and the anti-calretinin antibody is helpful for the differential diagnosis between mesothelial cells and not only serous type, but also mucinous, endometrioid and clear cell types of ovarian cancer cells. ———— peritoneal cytology; reactive mesothelial cell; ovarian carcinoma; Ber-EP4; calretinin

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Cytologic examination of peritoneal fluids is an important tool in detecting intraperitoneal dissemination of genital tract cancers. Ovarian cancer patients are well-known to show the highest percentage of malignant peritoneal cytology, which reflects clinical stage, intraperitoneal metastasis and patient survival (Keettel et al. 1974; Zuna and Behrens 1996). In many cases, however, the differentiation of reactive mesothelial cells (RM) from neoplasms in peritoneal fluids cannot always be made definitively on morphology alone (Fox 1993). Zuna and Behrens (1996) demonstrated that the sensitivity and specificity of cytologic examination of peritoneal fluids were 88% and 83%, respectively. Some studies have examined the criteria for distinguishing RM from adenocarcinoma cells, but the effectiveness of these criteria are limited, especially with respect to cytologic specimens (Covell et al. 1985; Mulvany 1996; Pisharodi et al. 1996; Weir and Bell 2001).

Immunohistochemical procedures have been widely utilized in aiding the differential diagnosis of numerous gynecological pathologies. However, no specific markers have been found to be diagnostic in differentiating RM from adenocarcinomas. Some of the markers that proved to be useful in separating RM from lung cancer cells have a different value in differentiating between RM and ovarian cancer cells (Ordoñez 1998). Therefore, the use of a panel of antibodies provides the highest degree of accuracy. Many studies have attempted to apply immunocytochemical staining in the differentiation of these two types of cells. However, large differences exist in the results of these published studies, regarding both the value of some of the markers and which markers should be included in the routine diagnostic panel for differentiating them (Delahaye et al. 1997; Fetsch et al. 1998; Ordoñez 1998; Lozano et al. 2001; Attanoos et al. 2002). In addition, previous studies examined only serous type but not other histological types of ovarian carcinoma cases. The aim of this investigation was to assess the practical value of markers for identifying mesothelial cells including calretinin, vascular cell adhesion molecule-1 (VCAM-1) and mesothelial cell antigen and of markers for identifying epithelial cells including carcinoembryonic antigen (CEA), CA-125, cytokeratin 18, epithelial membrane antigen (EMA), epithelial related antigen and epithelial antigen in distinguishing RM from various types of ovarian cancer cells. Our findings suggest that epithelial antigen and calretinin immunoreactivity are highly effective in differentiating RM from ovarian carcinoma than other carcinoma and mesothelial markers, when employed in immunohistochemical study. We then confirmed the effectiveness of these antibodies in the immunocytochemical diagnosis of RM and ovarian cancer cells in cytologic specimens.

Materials and Methods

Forty-nine cases of ovarian carcinoma (serous, n = 22; mucinous, n = 10; endometrioid, n = 7; clear cell, n = 10) and fifteen normal mesothelial tissues were retrieved from surgical pathology files at Tohoku University Hospital, Sendai, Japan. Clinicopathological findings for these patients were retrieved by review of patient charts. None of the patients described in this study had received preoperative chemotherapy and/or hormonal therapy or pelvic radiation. None of the patients used oral contraceptives. The lesions were classified according to the Histological Typing of Female Genital Tract Tumors by WHO (Tavassoli and Devilee 2003). All specimens were routinely processed (i.e., 10% formalin fixed for 24 to 48 hours), paraffin embedded, and thin sectioned (3 μm).

Immunohistochemical analysis was performed employing the streptavidin-biotin amplification method using a Histofine Kit (Nichirei, Tokyo), as previously described (Suzuki et al. 1994). Table 1 shows a summary of the immunomarkers used, the suppliers and specifications. All antibodies used in this study are mouse monoclonal antibodies, except for an anti-calretinin polyclonal antibody. The sections were pretreated with trypsin for Ber-EP4, proteinase K for anti-CEA, or pronase for anti-calretinin, or treated in citrate buffer at pH 6.0 in a microwave oven for 30 min for anti-CA125. The sections for MOC-31, anti-cytokeratin 18 and anti-VCAM-1 stainings were pretreated by autoclaving in citrate buffer (pH 6.0) at 121°C for 10 min. The antigen-antibody complex was visualized with 3,3´- diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris-HCl buffer [pH 7.6], and 0.006% H_2O_2), and counterstained with hematoxylin. Suitable positive control tissues were used to confirm antibody specificity. As a negative control, nor-

TABLE 1. *Characteristics of primary antibodies employed in immunohistochemistry*

Antibody to:	Clone	Source	Dilution	Antigen retrieval
Epithelial cell markers				
Epithelial antigen	Ber-EP4	DakoCytomation*	1 : 25	Trypsin
EMA	E29	DakoCytomation	1 : 50	
Epithelial related antigen	MOC-31	DakoCytomation	1 : 50	Autoclave
Cytokeratin18	DC10	DakoCytomation	1 : 50	Autoclave
Tumor markers				
CA125	M11	DakoCytomation	1 : 20	Microwave
CEA	II-7	DakoCytomation	1 : 50	Proteinase K
Mesothelial cell and mesothelioma markers				
Mesothelial cell	HBME-1	DakoCytomation	1 : 50	
VCAM-1	1.4C3	DakoCytomation	1 : 50	Autoclave
Calretinin	-	Swant**	1 : 8000	Pronase

* DakoCytomation, Glostrup, Denmark ; ** Swant corp., Bellinzona, Switzerland.

EMA, epithelial membrane antigen; CEA, carcinoembryonic antigen; VCAM-1, vascular cell adhesion molecule-1.

mal rabbit or mouse IgG was used instead of the primary antibodies. No specific immunoreactivity was detected in these sections.

In addition, we examined a total of 43 cytologic specimens of ascites or peritoneal lavage fluid retrieved from the cytology files at Tohoku University Hospital, Sendai, Japan. These specimens consisted of 20 RMs and 23 adenocarcinomas of the ovaries (serous, $n = 18$; mucinous, $n = 1$; endometrioid, $n = 2$; clear cell, $n = 2$). All primary tumors were confirmed by histology. Cytologic specimens consisting of RMs were retrieved from the cases of ovarian carcinoma. The diagnoses of cytologic specimens were confirmed by board-certified cytopathologists (Sasano H. and Ito K). These cases are different from previous ones used in immunohistochemical analysis, in order to avoid bias. The collected cells obtained from ascites or peritoneal lavage fluid were fixed onto glass slides using an auto-smear method (Sakura, Inc., Tokyo). All slides were Papanicolaou-stained and reviewed to ensure that they were adequate for diagnosis. For immunocytochemistry, one representative Papanicolaou-stained smear was selected in each case and destained with 1% hydrochloric acid in 95% ethanol. We then performed all immunocytochemical procedures with primary antibodies, anti-calretinin and Ber-EP4, employing the streptavidin-biotin amplification method using a Histofine Kit. Staining was performed following either our procedure or the procedure proposed by Doglioni et al. (1996; Okamoto 1996).

The immunostaining of tumor and/or mesothelial cells was evaluated for cytoplasmic staining. Calretinin expression was demonstrated by nuclear and cytoplasmic immunostaining. Staining for Ber-EP4 and the anti-calretinin antibody was considered positive if any number of tumor and/or mesothelial cells showed positive staining. The slides were assessed by two of the authors (K.I. and S.O.) Statistical analysis was performed using Stat View 5.0 (SAS Institute Inc., Cary, NC, USA) software. With the association between immuno-positive and -negative epithelial antigen and calretinin as explanatory variables, the judgment of whether ovarian carcinoma or mesothelial cells were detected as a dependent variable was evaluated with correlation coefficient (r) and regression equation.

RESULTS

Results of carcinoma and epithelial cell markers in 49 ovarian carcinomas and fifteen normal mesothelial tissues are summarized in Table 2. More than 90% of ovarian carcinoma cells reacted positively with epithelial antigen, EMA, epithelial related antigen and cytokeratin 18. On the other hand, EMA, epithelial related antigen and cyto-

TABLE 2. *Immunoreactivity for epithelial and tumor markers in tissue specimens*

	Epithelial related					
	Epithelial antigen	EMA	Antigen	CK	CA125	CEA
Serous (22)	86% (19/22)	100% (22/22)	100% (22/22)	100% (22/22)	100% (22/22)	27% (6/22)
Mucinous (10)	100% (10/10)	80% (8/10)	100% (10/10)	90% (9/10)	50% (5/10)	90% (9/10)
Endometrioid (7)	100% (7/7)	100% (7/7)	100% (7/7)	100% (7/7)	86% (6/7)	29% (2/7)
Clear (10)	90% (9/10)	100% (10/10)	100% (10/10)	100% (10/10)	100 (10/10)	10% (1/10)
Positivity (%) (Positive No./Total No.)	92% (45/49)	96% (47/49)	100% (49/49)	98% (48/49)	88% (43/49)	37% (18/49)
Normal mesothelial tissue section (15)	0% (0/15)	20% (3/15)	33% (5/15)	100% (15/15)	100% (15/15)	7% (1/15)

EMA, epithelial membrane antigen; CK, Cytokeratin 18; CEA, carcinoembryonic antigen.

keratin 18 immunoreactivities were demonstrated in 20%, 33% and 100% of mesothelial cells, respectively. Only epithelial antigen immunoreactivity was not detected in these cells.

Results of mesothelial cell markers in ovarian carcinomas and others are summarized in Table 3. Sixty-seven to 100% of mesothelial cells demonstrated immunoreactivity of mesothelial cell antigen, VCAM-1 and calretinin. In comparison, mesothelial cell antigen, VCAM-1 and calretinin immunoreactivity was expressed in 84%, 39% and only 6% of ovarian carcinoma cells, re-

TABLE 3. *Immunoreactivity for mesothelial cell and mesothelioma markers in tissue specimens*

	Mesothelial antigen	VCAM-1	Calretinin
Serous (22)	100% (22/22)	68% (15/22)	5% (1/22)
Mucinous (10)	30% (3/10)	20% (2/10)	10% (1/10)
Endometrioid (7)	86% (6/7)	0% (0/7)	14% (1/7)
Clear (10)	100% (10/10)	20% (2/10)	0% (0/10)
Positivity (%) (Positive No./Total No.)	84% (41/49)	39% (19/49)	6% (3/49)
Normal mesothelial tissue section (15)	100% (15/15)	67% (10/15)	100% (15/15)

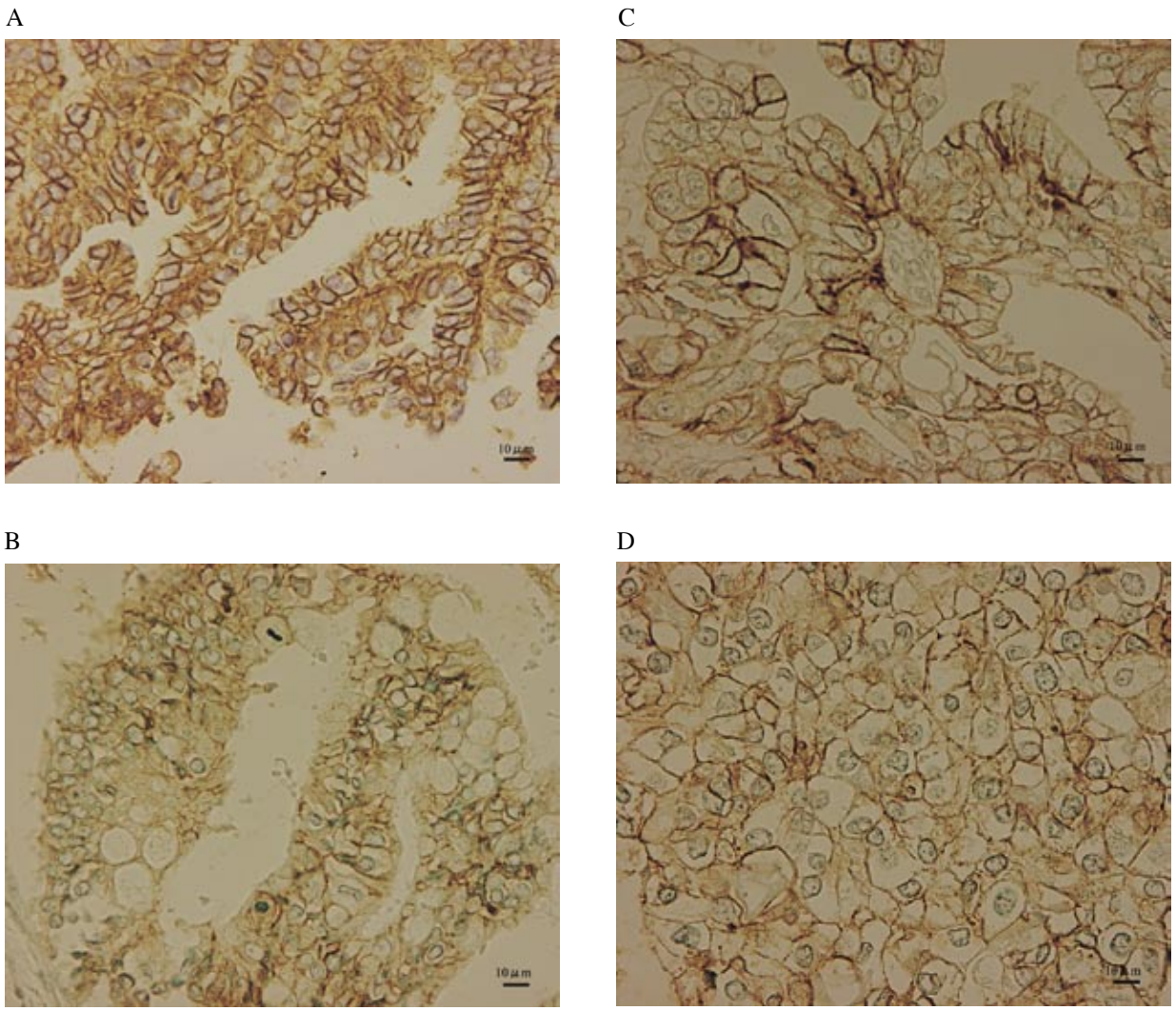

Fig. 1. Immunohistochemistry for epithelial antigen in surgical resections of: A, serous adenocarcinoma; B, mucinous adenocarcinoma; C, endometrioid adenocarcinoma; D, clear cell adenocarcinoma. Brown staining was detected in the cell membranes of all specimens. Original magnification × 400.

spectively. These results suggest that epithelial antigen and calretinin immunoreactivity are highly effective in differentiating RM from ovarian carcinoma than other epithelial and mesothelial markers when employed in immnohistochemical study (Figs. 1, 2 and 3). We therefore examined the effectiveness of these antigens (epithelial antigen and calretinin) in the diagnosis of RM and ovarian carcinoma cells in cytologic specimens (Fig. 4). Immunoreactivity for epithelial antigen in cytologic specimens is summarized in Table 4. The sensitivity and specificity of Ber-EP4 in these specimens were 100% and 90%, respectively. Immunoreactivity for calretinin in cytologic specimens is summarized in Table 5. The sensitivity and specificity of the anti-calretinin antibody in these specimens were 90% and 91%, respectively. Only two (one is serous type and another is clear cell type) of 23 ovarian carcinomas expressed immuno-reactive calretinin. Using multiple regression analysis with immuno-positive and -negative epithelial antigen and calretinin as explanatory variables, and the judgment of whether ovarian carcinoma or mesothelial cells were detected as a dependent variable, we determined the correlation coefficient to be $r = 0.938$, with a significance

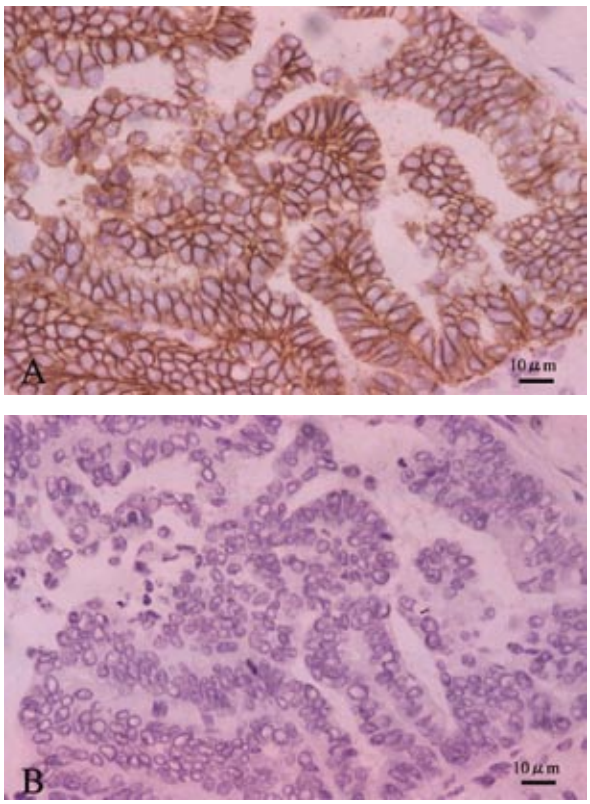

Fig. 2. Immunohistochemistry for epithelial antigen and calretinin in serial sections of serous ovarian carcinoma. A: Epithelial antigen immunoreactivity was predominantly detected in the cell membranes of cells. B: Calretinin immunoreactivity was negative. Original magnification × 400.

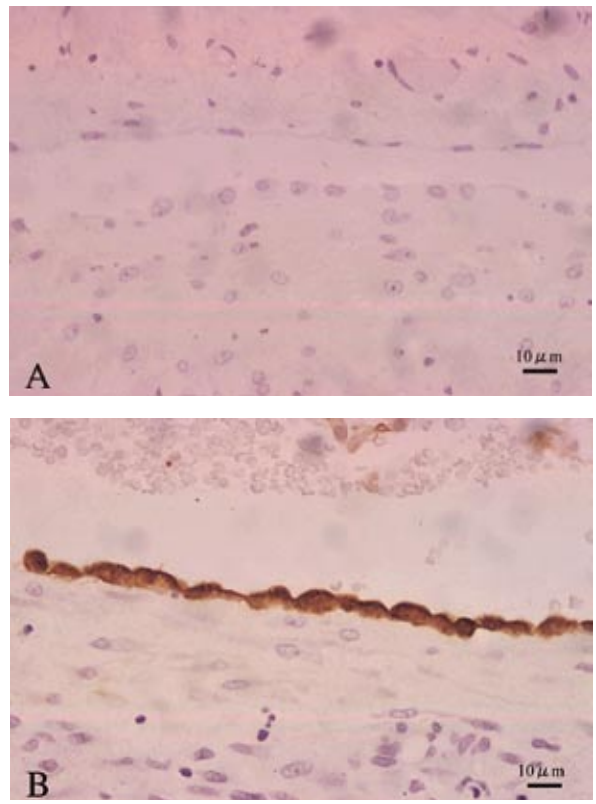

Fig. 3. Immunohistochemistry for epithelial antigen and calretinin in serial sections of a normal mesothelium. A: Epithelial antigen immunoreactivity was negative. B: Calretinin immunoreactivity was detected in both the nuclei and cytoplasm. Original magnification × 400.

level of $p < 0.0001$. These findings match well into an estimated multiple regression model (Table 6).

Discussion

The differential diagnosis between RM and adenocarcinoma in pleural and/or peritoneal effusion continues to be a diagnostic problem in routine cytology practice. Immunocytochemistry is considered useful as a diagnostic aid in this type of differential diagnosis. To date, a significant number of antibodies have been applied to differential diagnosis of cytology specimens from pleural and/or peritoneal effusion, with various degrees of efficacy. However, the usefulness of immunocytochemistry in peritoneal cytology has been less well explored than in pleural effusions. Studies of peritoneal immunocytochemistry with respect to the origin of ovarian carcinoma are markedly limited. Although ovarian cancer patients have the highest percentage of malignant peritoneal cytology, which represents the clinical stage, intraperitoneal metastasis and patient survival, the number of the patient specimens was small and insufficient (Khoury 1990; Ordoñez 1998a; Lozano et al. 2001; Attanoos et al. 2002). Furthermore, these studies examined only serous type but not other histological types of ovarian carcinoma cases. The differentiation of RM from ovarian carcinoma is very important for management of ovarian cancer patients. To our knowledge, the present study is the first report that summarizes data from the largest number and on various types of ovarian cancer cases demonstrat-

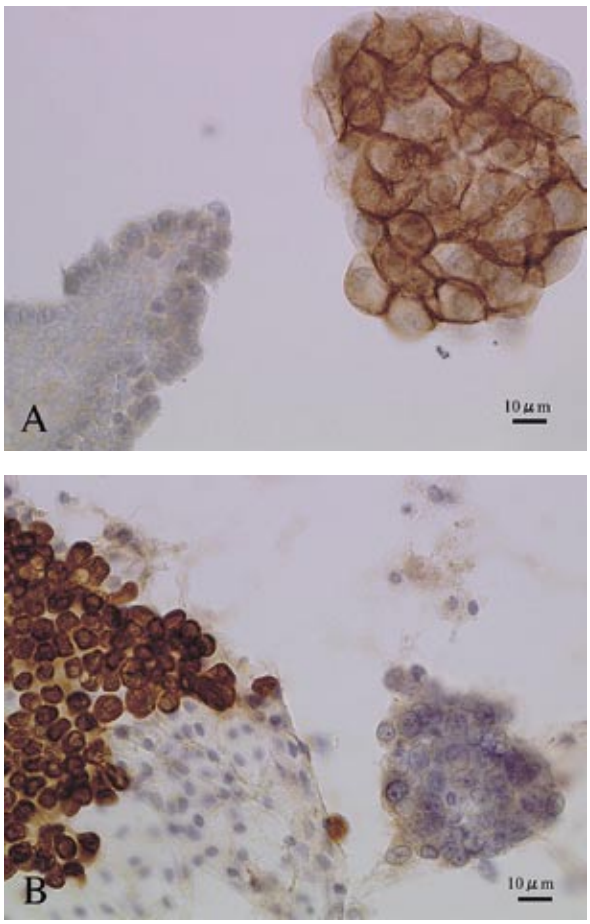

Fig. 4. A: Immunostaining for epithelial antigen in an ascites sample diagnosed as positive following cytodiagnosis. The cell membranes of this serous ovarian carcinoma cell are intensely stained, whereas normal mesothelial cells are negative for epithelial antigen immunostaining. B: Immunostaining for calretinin in an ascites sample diagnosed as positive following cytodiagnosis. Mesothelial cells are stained positive for calretinin, whereas ovarian carinoma cells are negative. Original magnification × 400.

ing the usefulness of immunocytochemistry in the differentiation of RM from ovarian carcinoma.

In our immunohistochemical study, epithelial antigen and calretinin were found to be effective discriminant markers in distinguishing RM from ovarian carcinomas, compared to other epithelial and mesothelial markers in surgical pathology specimens described previously (Sato et al. 2000). Ber-EP4 is a monoclonal antibody that recognizes an epitope present on 2 glycopeptides of 30 kDa and 34 kDa molecular weight, respectively (Latza et al. 1990). This recognized antigen is considered to be present in human epithelial cells and carcinomas but almost absent in the mesothelium (Latza et al. 1990; Sheibani et al. 1991; Ordoñez 1999). In 1990, Latza et al. reported epithelial antigen reactivity in 142 (98.6%) of 144 carcinomas of various organs. However, no reactivity was detected in 88 nonepithelial tumors, including 14 mesotheliomas. In another report, Sheibani and colleagues detected epithelial antigen immunoreactivity in 72 (87%) of 83 adenocarcinomas of various organs but in only one (1%) of 115 mesotheliomas (Sheibani et al. 1991). These studies appear to suggest that epithelial antigen could be of use in the differentiation of mesothelium from adenocarcinomas. According to the literature, epithelial antigen immunoreactivity depends on the primary site of the tumor (Ordoñez 1998b, 1999). Several studies have reported epithelial antigen immunoreactivity in ovarian carcinoma. Ordoñez (1998a) found that epithelial antigen reactivity was detected in all 30 serous ovarian carcinomas but in only four (11%) of 35 mesotheliomas. In addition, Attanoos et al. (2002) reported that epithelial antigen showed 95% sensitivity and 91% specificity in 32 diffuse peritoneal mesotheliomas,

TABLE 4. *Immunoreactivity for epithelial antigen in cytologic specimens*

	Ov. ca.	Mesothelial cell
Epithelial antigen positive (No.)	23	2
Epithelial antigen negative (No.)	0	18
Sensitivity: 100%	Specificity: 90%	

Ov. ca., ovarian carcinoma cells.

TABLE 5. *Immunoreactivity for calretinin in cytologic specimens*

	Ov. ca.	Mesothelial cell
Calretinin positive (No.)	2	18
Calretinin negative (No.)	21	2
Sensitivity: 90%	Specificity: 91%	

Ov. ca., ovarian carcinoma cells.

TABLE 6. *Multiple regression analysis*

	Ov.ca.	Mesothelial cell
Epithelial antigen positive calretinin positive (No.)	2	2
Epithelial antigen positive calretinin negative (No.)	21	0
Epithelial antigen negative calretinin positive (No.)	0	17
Epithelial antigen negative calretinin negative (No.)	0	1
	23	20

Ov.ca., ovarican carcinoma cells.

The correlation coefficient between epithelial antigen and calretinin reactivity was $r = 0.938$, with a significance level of $p < 0.0001$.

20 serous papillary ovarian carcinomas and three primary peritoneal serous papillary carcinomas. Results from our present study are also consistent with these previous reports above. Epithelial antigen immunoreactivity was detected not only in serous ovarian carcinomas, but also in all mucinous, endometrioid and clear cell ovarian carcinomas.

Calretinin, which is a 29-kDa calcium-binding protein normally present in neurons of the central and peripheral nervous system, has recently been recognized as an immunohistochemical marker of reactive mesothelium and malignant mesothelioma in tissue sections (Andressen et al. 1993; Doglioni et al. 1996). In 1996, Doglioni et al. reported calretinin immunoreactivity in all of 44 mesotheliomas, but only focally in 28 (10%) of 294 adenocarciomas of various organs including one (6%) of 16 serous papillary ovarian carcinomas. Ordoñez (1998a), who used a different commercial antibody, reported that all 35 mesotheliomas demonstrated calretinin expression, whereas only three (10%) of 30 serous papillary ovarian carcinomas expressed calretinin immunoreactivity. The distribution of calretinin immunoreactivity has recently been suggested to be useful as the only nuclear reactivity marker that is highly specific for the mesothelium (Cury et al. 2000; Attanoos et al. 2002). These studies demonstrated rates of immunoreactivity for normal and neoplastic mesothelium ranging from 80% to 100%, with only rare adenocarcinomas exhibiting nuclear immunoreactivity. Interpreting only nuclear immunoreactivity as being unequivocally positive, a study by Attanoos et al. (2002) recently reported that calretinin was identified as the most sensitive (88%) and specific (100%) mesothelial marker in the distinction of peritoneal mesothelioma from

serous papillary ovarian carcinoma with.

CEA has been widely applied in immunohistochemistry due to its ability to distinguish adenocarcinomas from reactive and neoplastic mesothelium. Although CEA is considered to be highly specific, it has a relatively low sensitivity in serous ovarian carcinomas (Brown et al. 1997; Ordoñez 1998a). Our findings were consistent with those previous reports. Results from our study described the immuno-reactive expression of immunoreactivity of epithelial related antigens, cytokeratin 18, EMA and CA125 in the great majority of ovarian carcinomas and mesothelial tissues, a finding consistent with previous reports (Delahaye et al. 1991, 1997; Bateman et al. 1997; Ordoñez 1998a; Attanoos et al. 2002). These results suggest that these markers are not necessarily helpful in distinguishing adenocarcinomas of the ovary from a reactive and/or neoplastic mesothelium. VCAM-1 is a cytokine-induced adhesion molecule which recognizes ligands that are abundantly present on leukocytes. Ruco et al. (1996) recently reported that VCAM-1 was detected in 14 of 16 malignant mesotheliomas and in only one of 58 epithelial malignant tumors. However, in our study, VCAM-1 was expressed in ovarian carcinomas, especially in the serous papillary type. HBME-1 was generated using a suspension of human mesothelioma cells from patients diagnosed with malignant epithelial mesothelioma. No mesothelial antigen reactivity has been detected in normal cells other than mesothelial cells, but this antibody has been reported to react with adenocarcinomas in some organs (Fetsch and Abati 2001). Our results have confirmed that mesothelial antigen immunostaining has no practical value in distinguishing between these two tumors.

From our findings we conclude that epithelial antigen and calretinin are useful discriminant markers in the mesothelia for not only serous but also mucinous, endometrioid and clear cell types of ovarian carcinomas. We also examined the effectiveness of these antibodies (Ber-EP4, anti-calretinin) in the diagnosis of mesothelial cells and ovarian cancer cells in cytologic specimens. Immunocytochemical results from the present study suggest that the combined immunostaining for Ber-EP4 and the anti-calretinin antibody is helpful for the differential diagnosis between mesothelial cells and not only serous type, but also mucinous, endometrioid and clear cell types of ovarian cancer cells in cytologic specimens.

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