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Heidi S. Berner · Ben Davidson · Aasmund Berner
Bjørn Risberg · Jahn M. Nesland

Differential expression of CD44s and CD44v3-10 in adenocarcinoma cells and reactive mesothelial cells in effusions

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Abstract The detection of malignant cells in serous effusions obtained from patients diagnosed with cancer marks the presence of metastatic disease and is associated with a poor outcome. The purpose of this study was to evaluate the role of CD44s and CD44v isoforms in the distinction between mesothelial cells and malignant epithelial cells in effusions. Fifty-nine fresh pleural and peritoneal effusions were studied. These consisted of 41 specimens from patients with known gynecological neoplasms, 9 from patients diagnosed with breast adenocarcinoma, and 9 effusions from patients with various nongynecological malignancies or tumors of unknown origin. Forty-three effusions contained malignant/atypical epithelial cells, and 16 effusions were diagnosed as reactive. Three effusions contained exclusively malignant cells. Specimens were stained with anti-CD44s, v3, v5, v6, v7 and v3-10. The presence of staining in cancer cells, benign mesothelial cells and lymphocytes was evaluated. CD44s immunoreactivity was seen in 10 of 43 (23%) cases in malignant/atypical epithelial cells and in 53 of 56 (94%) cases in benign cells. In contrast, CD44v3-10 was seen in 23 of 43 (55%) cases in malignant/atypical epithelial cells and in 3 of 56 (6%) cases in benign cells. We advocate the use of CD44s and CD44v3-10 immunostaining in diagnostic evaluation of difficult serous effusions.

Key words Adenocarcinoma cell · Mesothelial cells · Effusions · Immunohistochemistry

Introduction

Malignant neoplasms are characterized by their ability to infiltrate local tissues and metastasize to distant organs. The prevalent mechanism for the spread in a number of neoplasms is the extension of tumor cells from the primary tumor into the pleural and peritoneal cavities [3, 22]. In routine diagnostic work it is sometimes difficult to distinguish between benign mesothelial cells and adenocarcinoma cells in cytological preparations by the application of morphological criteria alone. However, both immunohistochemical and molecular biological examinations can be useful adjuncts [7, 9].

CD44 is a transmembrane glycoprotein which is widely expressed in hematopoietic and mesodermal cells [18]. The CD44 gene is composed of at least 20 exons, 10 of which are constitutively expressed in almost all cell types, to produce a heavily glycosylated ~85-kDa isoform called the standard form (CD44s) [25]. The remaining exons can be alternatively spliced in various combinations, and their products are incorporated into the polypeptide backbone encoded by CD44s.

Multiple functions are attributed to the various isoforms of CD44, such as lymphocyte homing, hematopoiesis, inflammation, wound healing, embryonic development and apoptosis [13, 15, 23, 28, 31]. CD44 also plays a part in tumor cell differentiation, invasion and metastasis. CD44 is the principal receptor for hyaluronic acid (HA) [33], expression of which is one of the most characteristic findings in malignant mesothelioma. Three different binding sites have been identified for CD44 binding to HA: constitutive, inducible and nonbinding [18].

CD44 gained attention as a marker of metastasis because metastatic and nonmetastatic tumors differ in the expression of CD44v isoforms [10, 13, 34]. CD44v isoform expression has been demonstrated in many types of human cancers. Expression of CD44v is higher in non-Hodgkin lymphoma [29], breast cancer [4, 14, 17] and colon cancer [20] than in corresponding normal tissues. Furthermore, CD44v expression has been correlated with lymph node metastases in colon cancer [20] and breast cancer [4, 14, 16]. On the other hand, in uterine cancer [24] and in prostate cancer [16], expression of CD44v is lower than in corresponding normal tissues and reduced CD44v expression is associated with tumor metastasis.

H.S. Berner (✉) · B. Davidson · A. Berner · B. Risberg
J.M. Nesland
Department of Pathology, The Norwegian Radium Hospital, University of Oslo, Montebello, N-0310 Oslo, Norway
e-mail: hberner@odont-uio.no
Tel.: +47-22934194, Fax: +47-22730164

Previous studies of CD44 expression in ovarian carcinoma have produced conflicting results. Roderiguez et al. demonstrated elevated expression of CD44v isoforms [6, 21]. In contrast, Sliutz et al. [26] and Speiser et al. [27] reported minimal expression of CD44v isoforms. These apparently contradictory findings for various malignant tumors suggest that the function of CD44 isoform molecules possibly differ from organ to organ and in different cell types. The aim of this study was to establish whether CD44 isoforms have diagnostic utility in differentiating benign reactive mesothelial cells from metastatic carcinoma cells in effusions.

Materials and methods

Materials

The materials consisted of 59 consecutive fresh nonfixed peritoneal and pleural effusions submitted to the Section of Cytology, 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 age ranged from 16 to 86 years. The material included 41 samples from patients with known gynecological neoplasms, 9 samples from breast cancer patients, and 9 from patients with various nongynecological malignancies or tumors of unknown origin. The distribution of cases according to tumor origin and histological diagnosis is shown in Table 1. All samples were previously studied using five epithelial markers [9].

Control cases consisted of (a) formalin-fixed and paraffin-embedded cell blocks of two breast carcinoma cell lines (SK-BR-3, MDA-MD-231) and (b) biopsies from 13 specimens from malignant mesotheliomas, 11 of which were classified as epithelioid, 1 as sarcomatous and 1 as of the combined type. (c) In 10 of the cases (9 of ovarian carcinoma and 1 of endometrial carcinoma), formalin-fixed, paraffin-embedded material from primary tumors were also immunostained and the results compared with those for CD44v6.

Specimen protocol

All specimens were received as fresh nonfixed effusions, and there was a volume range of 20–2000 ml. The specimens were centrifuged for 10 min at 2000 rpm. The resulting pellet was used for the preparation of four cytological smears (two alcohol-fixed Papanicolau-stained and two air-dried Diff-Quick-stained) and a formalin-fixed and paraffin-embedded cell block. The remaining material was frozen in RPMI medium containing DMSO. Cell blocks were prepared using Shandon Lipshaw cytoblock kit (Shandon, Pittsburgh, Pa.).

Morphology

Cytologic smears from all samples were evaluated by two experienced cytopathologists (B.R., A.B.) 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 by two experienced cytopathologists. The diagnostic criteria employed were according to guidelines by Bedrossian [5]. All cases were subsequently reviewed by three of the authors (B.D, B.R, A.B.) before the evaluation of immunostaining results. For 10 cases in which discrepant morphological diagnoses were obtained a discussion was held, in which the slides were re-evaluated and a consensus diagnosis was always achieved.

Table 1 Case distribution according to tumor origin

Diagnosis	No. of cases
Ovary: adenocarcinoma	31
Ovary: borderline tumor	1
Ovary: benign cyst	3
Uterus: endometrial adenocarcinoma	4
Uterus: carcinosarcoma	3
Cervix: adenocarcinoma	1
Primary peritoneal carcinoma	4
Breast: adenocarcinoma	9
Colon: adenocarcinoma	2
Unknown	1
Total	59

Antibodies

The following monoclonal antibodies were used: BMS 113 against an epitope encoded by a CD44s region, BMS144 against an epitope encoded by exon v3, BMS 115 against an epitope encoded by exon v5, BMS 125 against an epitope encoded by exon v6, and BMS 117 against an epitope encoded by exon v7. The polyclonal antibody BMS 124 against an epitope encoded by exons v3–v10 was also used. All antibodies were obtained from Bender MedSystems (Vienna, Austria). Working dilutions were 1:20 for BMS113; 1:50 for BMS114, BMS115, BMS 117, and BMS 124; and 1:100 for BMS 125.

Immunohistochemistry

Following routine deparaffinization, sections were microwave treated in 10 mM citrate buffer, pH 6.0 for antigen retrieval. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min and preincubated with 20% normal serum diluted in 1% bovine serum albumin (BSA) at room temperature for 20 min. Separate sections were then incubated with the primary antibodies at room temperature for 1 h. This was followed by incubation with biotinylated secondary antibody at room temperature for 30 min and thereafter incubation with horseradish-peroxidase-conjugated avidin-biotin complex at room temperature for 1 h. Immunostaining was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB)/H_2O_2 substrate. After incubation with horseradish-peroxidase-conjugated avidin-biotin complex and (DAB)/H_2O_2, cells were washed twice for 10 min using Tris-HCl buffer pH 7.6. Cells were counterstained with hematoxylin. Two breast carcinoma cell lines (SK-BR-3, MDA-MB-231) were tested as positive controls for all antibodies, using cell blocks that were prepared using the above procedure. MDA-MB-231 and SK-BR-3 were chosen for CD44s, v3 and v6 and CD44v5, v7 and v3–10, respectively, and used in every staining reaction. One paraffin-embedded specimen of lobular carcinoma in situ was tested and used as a negative control for all antibodies and applied in every staining reaction. The control stainings were satisfactory each time. Control specimens of malignant mesothelioma were stained with CD44s and v3–10.

Interpretation of staining results

The presence of staining in malignant/suggestive cells, benign mesothelial cells, and lymphocytes was clarified after morphological evaluation. A minimum of 200 cells (if available) was evaluated to determine the percentage of stained cells. All morphology-negative immunohistochemistry-positive cases were re-evaluated morphologically, in order to verify the existence of a malignant cell population. Both membranous and cytoplasmic staining were evaluated.

Table 2 Immunohistochemical staining results

Class	Antibody	Total positive
Malignant/atypical epithelial cells	CD44s	12/43 (23%)
	CD44v3–10	23/43 (55%)
Benign mesothelial cells	CD44s	53/56 (94%)
	CD44v3–10	3/56 (6%)
Lymphocytes	CD44s	28/46 (61%)
	CD44v3–10	0/46 (0%)
Malignant mesotheliomas	CD44s	9/13 (69%)
	CD44v3–10	5/13 (38%)

Results

Forty-three of 59 cases were classified as malignant and 16 as benign in the morphological evaluation of cytological smears and cell block sections. Benign mesothelial cells were detected in all reactive samples, and also in 40 of 43 cases classified as malignant or suggestive of malignancy. Lymphocytes were present in 46 of 59 effusions.

Immunostaining results with anti-CD44s (Fig. 1A) and CD44v3–10 (Fig. 1B) for malignant/suggestive cells, benign mesothelial cells, lymphocytes and control cases are shown in Table 2. The main antigenic reactivity in infiltrating cells with CD44-specific antibodies was seen on the cell membrane. A variable number of cells stained with anti CD44v3–10 revealed additional cytoplasmic staining, most probably reflecting CD44 molecules in the Golgi apparatus.

CD44s immunoreactivity was detected in malignant or atypical epithelial cells in 10 of 43 cases (23%) and in benign cells in 53 of 56 cases (94%). CD44v3–10 membranous staining in malignant or atypical epithelial cells was detected in 23 of 43 cases (55%) and in benign cells in 3 of 56 cases (6%). Focal expression of CD44v3, v5, v6 and v7 in malignant cells was seen in isolated cases (not shown). Immunoreactive lymphocytes were detected in 28 of 46 (61%) effusions using anti-CD44s, but were uniformly negative for CD44v3–10.

CD44v6 immunoreactivity was detected in 5 of 9 ovarian cancers and in the single case of endometrial cancer. None of the corresponding effusions showed immunoreactivity for CD44v6.

Atypical or malignant cells were found in cell block sections in 3 cases (6%) that were interpreted as benign in cytological smears. These atypical cells were uniformly negative for CD44s, whereas the surrounding benign cells expressed CD44s intensely. Malignant cells in 1 of the above-mentioned specimens showed immunoreactivity for CD44v3–10 (Fig. 2). CD44s positivity was observed in 8 epitheloid and 1 sarcomatous mesothelioma (69%). The immunostaining was focal in 1 epitheloid mesothelioma but diffuse in the others. None of the control mesotheliomas showed diffuse immunoreactivity for CD44v3–10. However, in 4 epitheloid and 1 sarcomatous mesothelioma (38%) focal CD44v3–10 immunostaining was revealed.

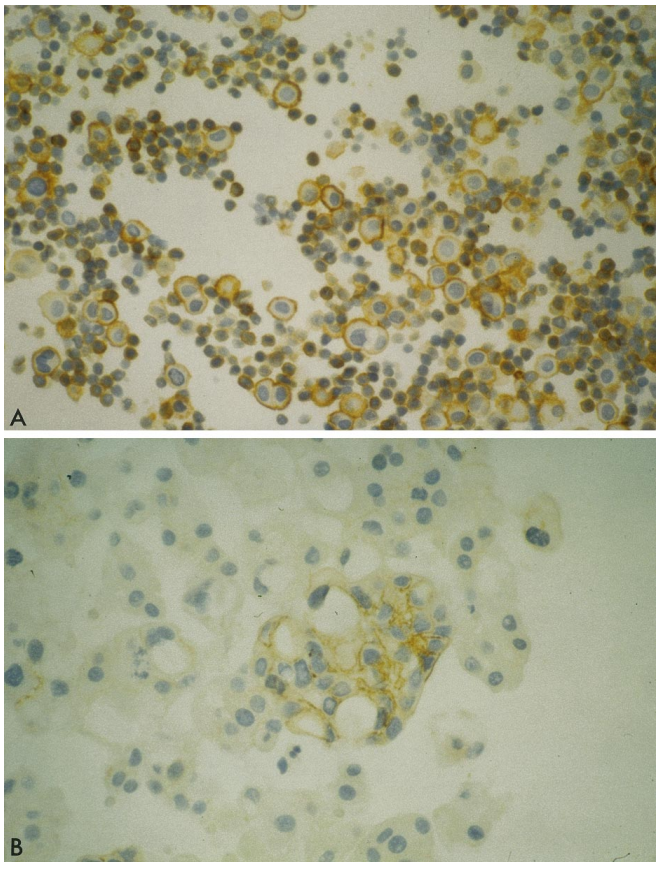

Fig. 1 **A** Membranous immunostaining for CD44s of cells of mesothelial origin in a case of ovarian carcinoma. **B** Membranous immunostaining for CD44v3–10 of malignant/atypical epithelial cells of a cervical adenocarcinoma

Discussion

In the present study, the role of six antibodies for different CD44 isoforms was evaluated for the distinction of benign mesothelial cells from metastatic carcinoma cells in effusions. A significantly greater expression of CD44s in benign mesothelial cells (94%) than in adenocarcinomas (23%) was demonstrated. The tendency of CD44s to be expressed more readily in benign mesothelial cells than in adenocarcinomas has also been shown in previous studies of reactive mesothelial hyperplasias and adenocarcinomas in tissue sections [1, 2] and in malignant effusions [11].

Based on cell block morphology and CD44 immunostaining, 3 additional cases were interpreted as malignant. This demonstrates the value of immunohistochemistry as an adjunct to morphological diagnosis of malignant effusions, and the beneficial use of CD44s and CD44v3–10 as markers of benign and malignant cells, respectively. The detection of malignant cells and their differentiation from reactive mesothelial cells in the study cohort have been described elsewhere [3]. In the latter study, five epithelial markers, i.e. Ber-EP4, carcinoembryonic antigen (CEA), CA-125, BG8 (Lewis' antigen) and B72.3 (TAG-72) were

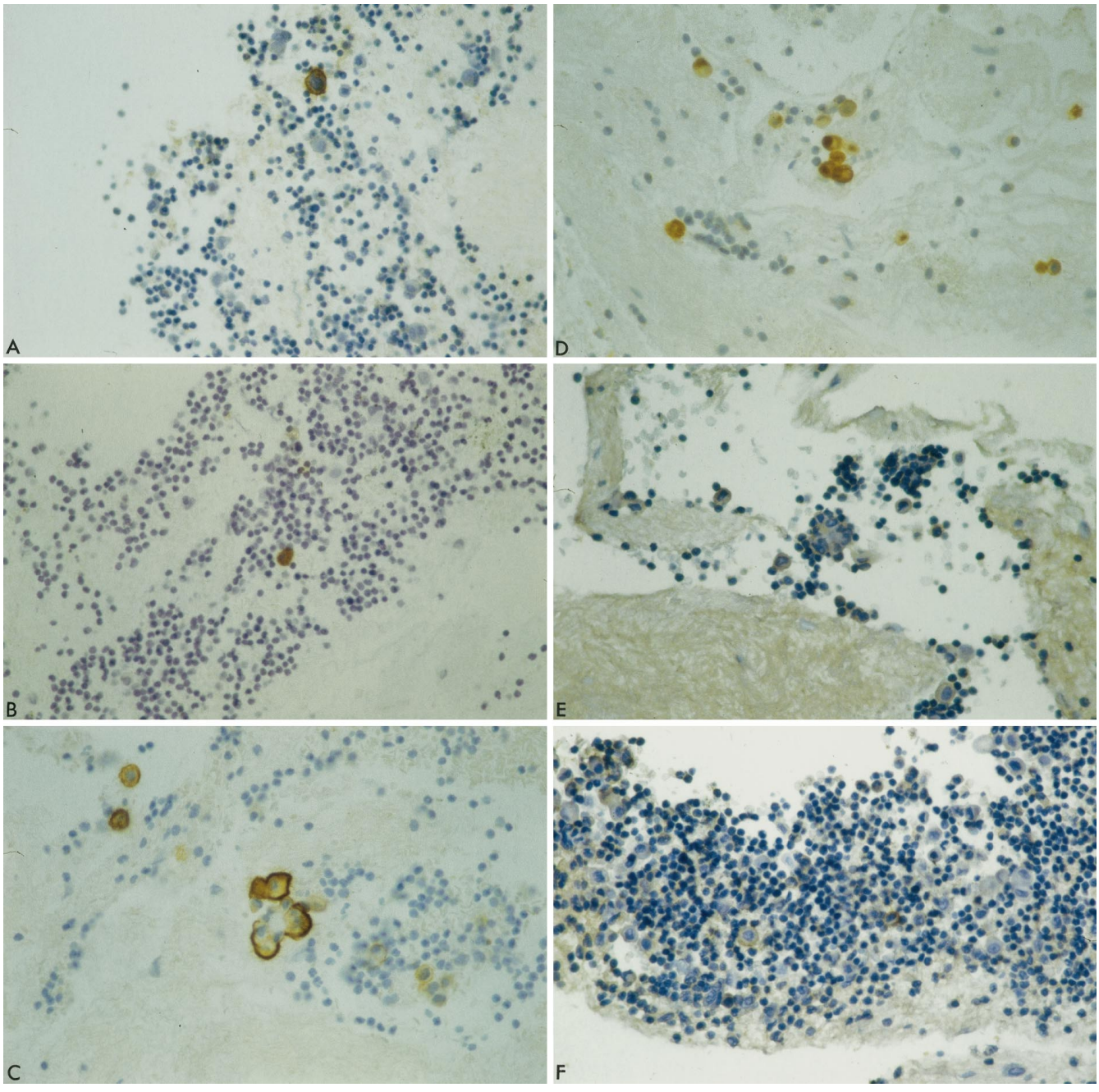

Fig. 2A–F Immunohistochemical findings for **A** Ber-EP4, **B** CEA, **C** CA-125, **D** calretinin, **E** CD44v3–10, and **F** CD44s in a case of colon adenocarcinoma

employed. E-Cadherin and calretinin were subsequently used for further characterization of the cohort (unpublished results). Thus, the division of cell populations into malignant and benign is established on both morphological criteria and results of immunohistochemistry.

To our knowledge, no studies of CD44v isoforms in effusions have been published to date. Our immunostaining results indicate that CD44v3–10 can be used in differentiation of benign mesothelial cells and adenocarcinoma cells in malignant effusions as adjuncts to cytological features. This agrees with a previous study reporting a correlation between the presence of CD44v3–10 and poor prognosis in 44 patients with primary ovarian carcinomas [32].

Isolated cases in our series showed focal expression of CD44v3, v5, v6 and v7, indicating that these antibodies are not useful in differentiation of benign mesothelial cells from metastatic carcinoma cells in effusions. However, in previous studies of lobular and ductal carcinomas of the breast, expression of CD44 splice variants containing protein sequences encoded by the variable exons v3, v5, v6 and v7 were reported [4, 8, 17, 30]. Moderate levels of CD44v3 and v7 and elevated levels of CD44v5 and v6 were also observed in lymph node me-

tastases compared with their respective primary carcinomas [8, 30]. So far, the role of CD44 splice variants in ovarian epithelial malignancy is not clear [6, 26, 32]. In agreement with our findings, some studies have reported a minimal expression of CD44v in ovarian carcinomas [26, 27], but on the other hand a high expression of CD44v4, v6 and v9 in ovarian carcinomas relative to normal tissue has also been reported [6, 26]. The present detection of CD44v6-positive cells in 6 of 10 paraffin-embedded primary tumors indicates that specific CD44 variants in effusions are down-regulated in effusions, compared with their corresponding primary tumors.

The epitope which is recognized by CD44s is encoded by exons that are also expressed in the various splice variants. The present study, however, shows that the immunohistochemical use of antibodies against both CD44s and v3–10 may be informative and useful in distinguishing benign mesothelial cells from adenocarcinomas. In a previous study Martegani and coworkers demonstrated that the immunoreactivity of some monoclonal antibodies directed to CD44 exon-specific epitopes can be impaired by the structural variability of the CD44 molecule [19]. This study showed that specific exon assortments and/or posttranslational modifications can mask CD44 exon-specific epitopes, and that glycosaminoglycans may have a critical role in determining the three-dimensional configuration of the molecule. It is also shown that exon v3 and 10 possess sites for the attachment for glycosaminoglycan side chains that may affect the binding of antibodies to CD44s [12].

In conclusion, 59 effusions were evaluated for the presence of malignant epithelial cells using monoclonal antibodies directed against CD44s, v3, v5, v6, v7 and CD44v3–10, respectively. Immunostaining for CD44s and v3–10 appears to give optimal results in tissue sections from effusions and can be used as an adjunctive diagnostic tool to morphology in the distinction between benign mesothelial cells and adenocarcinomas. Identification of CD44s points to a mesothelial cell origin, whereas CD44v3–10 immunoreactivity supports the diagnosis of adenocarcinoma. CD44v3, v5, v6 and v7 immunostaining appears to have no practical use in the differentiation of benign mesothelial cells from metastatic carcinoma cells in effusions.

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