Anti-EpCAM antibodies for detection of metastatic carcinoma in effusions and peritoneal wash

FABIANA PIRANI CARNEIRO[1-3], MARIA IMACULADA MUNIZ-JUNQUEIRA[2], MARCOS DE VASCONCELOS CARNEIRO[3], ÍSIS DE ARAÚJO OLIVEIRA[1], ALUÍZIO CARLOS SOARES[2], NATHÁLIA DE VARGAS HAAR[2], GUSTAVO HENRIQUE SOARES TAKANO[1], LEONORA MACIEL DE SOUSA VIANNA[1], GUILHERME DE CARVALHO CALDAS[1], DANILLO LEAL MARINHO VIEIRA[1], LÍGIA LINS FRUTUOSO[1], LARISSA MATOS RODRIGUES BRITO[1], RAFAEL VIEIRA MARTINS DE SIQUEIRA[1], AMANDA MOREIRA PARENTE[1], TERCIA MARIA MENDES LOUSA DE CASTRO[2], ISABELA PERES[1], LIANNA MARTHA SOARES MENDES[1], TATIANA KARLA DOS SANTOS BORGES[2], VÂNIA MORAES FERREIRA[2] and ANDREA BARRETTO MOTOYAMA[2]

[1]Pathological Anatomy Center of University Hospital of Brasília, Brasília 70840-050; [2]Pathology Department of Brasília University, Brasília 70910-900; [3]Catholic University of Brasilia, Brasília 71966-700, Brazil

Received September 23, 2018; Accepted May 21, 2019

DOI: 10.3892/ol.2019.10468

Abstract. Epithelial cell adhesion molecule (EpCAM) has been used as diagnostic/prognostic marker and therapeutic target. The aim of the present study was to compare immunoreactivity of antibodies against distinct epitopes in the ectodomain of EpCAM for detection of carcinoma from different primary sites and of different histological types in effusions and peritoneal wash. Two antibodies against epitopes in the EGF-like domain I (clones Moc-31 and Ber-EP4) and one antibody against the epitope in the cysteine-poor region (158210) of EpCAM were used (all commercially available). Independently of the clone used, EpCAM overexpression was observed in almost all samples when all the adenocarcinoma samples were analyzed together. By using Moc-31, EpCAM overexpression was observed in all samples of adenocarcinoma. Absence of EpCAM overexpression was observed in a few adenocarcinoma samples at some sites of tumor origin, including ovary, breast and stomach, when Ber-EP4 and 158210 were used. Regarding carcinomas aside from adenocarcinomas, histological types, such as squamous cell, urothelial and small cell carcinoma showed different degrees of EpCAM expression according to the antibody used. In squamous cell carcinoma, overexpression was observed only with the clone 158210. It was concluded that, overall, most samples of metastatic carcinoma from effusions showed overexpression of EpCAM. However, there are significant variations in its detection according to the primary site, histological type of the carcinoma and depending on the antibody used. Thus, the use of more than one type of anti-EpCAM antibody would increase the chance of its detection in metastatic carcinoma effusion.

Correspondence to: Mrs. Fabiana Pirani Carneiro, Pathological Anatomy Center of University Hospital of Brasília, Via L2 Norte, SGAN 604/605, Brasília 70840-050, Brazil
E-mail: fabianapirani@hotmail.com

Key words: effusions, epithelial cell adhesion molecule, immunocytochemistry, cytology, cancer, carcinoma

Introduction

Malignant effusion is the accumulation of cavity fluid due to the spread of malignant cells. It is a late-stage manifestation of cancer and is associated with poor prognosis. Since the most frequent cancer in effusions is adenocarcinoma, epithelial cell markers are among those used for the detection of cancer (1,2).

Epithelial cell adhesion molecule (EpCAM) is a 40 kDa transmembrane cell surface glycoprotein that is highly expressed in epithelial cancers and at lower levels in normal epithelia (3). Although it promotes homophilic cell-cell interactions, EpCAM modulates negatively cadherin-mediated cell adhesion resulting in an anti-adhesive effect during neoplasm development (4,5). Besides this, EpCAM was shown to play a role in cell proliferation. EpCAM's signaling mechanism suggests that EpCAM is subject to regulated intramembrane proteolysis and the cleaved intracellular domain is responsible for the induction of EpCAM's target genes (6,7). In gastric cancer, overexpression of EpCAM disrupts cell-cell contact, enabling the cellular migration that is required for metastasis (8).

Due to its frequent overexpression in carcinomas, EpCAM has been used as diagnostic/prognostic marker and therapeutic target (9). Liquid biopsy, for instance, a modern technology for cancer prognosis based on markers found in the peripheral blood, may be performed on EpCAM detection. Thus, a large number of antibodies against EpCAM have been used for

detection of carcinomas in effusion, blood and in biopsy and surgical specimens.

EpCAM is a polypeptide of 314 amino acids (aa) and contains a large extracellular domain (ectodomain) of 242 aa, a transmembrane domain of 23 aa, and an intracellular domain of 26 aa (10). EpCAM's extracellular domain contains a first motif with epidermal growth factor (EGF)-like repeats, a second motif that resembles a thyroglobulin (TY) type 1A repeat and a third motif that is cysteine free/poor and unrelated to any known molecule (3).

Most commercially available antibodies for carcinoma detection in blood and cavity fluids bind to the small N-terminal EF-like (EGF) domain (11). The clones Moc-31 and Ber-EP4 are the antibodies used for routine diagnosis of carcinomas in effusion. Both monoclonal antibodies recognize specific epitopes in the EGF-like domain.

By using monoclonal antibodies against different epitopes on the EpCAM ectodomain, different patterns of EpCAM expression would be expected. Parts of the protein might be absent, since EpCAM can be cleaved at multiple positions within its ectodomain (12).

To our knowledge, there are no published reports in which antibodies that recognize epitopes in the cysteine-poor region of EpCAM have been studied for detection of carcinoma in effusion and peritoneal wash. The aim of the present study was to compare immunoreactivity of antibodies with distinct epitopes in the ectodomain of EpCAM for detection of carcinoma from different primary sites and of different histological types in effusions and peritoneal wash.

Patients and methods

Patients and samples. Samples (n=55) of effusions (pleural, n=33; peritoneal, n=15; pericardial, n=5 and peritoneal wash n=2) were enrolled in the study. The samples were obtained at Department of Pathology of Brasilia University Hospital, Brazil, between 2015 to 2018. The study protocol was approved by the Human Ethics Review Committee of Brasilia University.

Diagnoses of carcinoma were established from clinical information, results of previous biopsy and results of cytology and immunocytochemistry.

All samples used here were fresh, and free of fixative or preservative solution. For cell block preparations, the method plasma/thromboplastin was used. The effusion/wash was centrifuged at 2,000 rpm for 2 min. Cell pellets were homogenized with 100 μl of plasma and 100 μl of tromboplastin (Stago®, Asnières sur Seine, France). After 2 min, the clots were fixed in formalin and subjected to usual histological processing. Sections of cell block on silanized microscope slides were stained with hematoxylin-eosin and used for immunocytochemistry.

For antigen retrieval, the slides were incubated for 45 min in a waterbath at 95-99˚C with citrate buffer pH 6.0. For blockade of endogenous tissue peroxide, the slides were immersed in 3% H_2O_2 solution at room temperature for 30 min. After washing with phosphate buffered saline (PBS), the slides were incubated with primary antibody overnight at 4˚C. The primary antibodies used are shown in Table I. A commercially available antibody against claudin 4 was used as additional positive control. After washing with PBS, the slides were incubated with a secondary antibody for 30 min at room temperature and subsequently with the streptavidin-peroxidase complex (Kit REVEAL-Biotin-Free Polyvalent DAB; Spring Bioscience, Inc., Pleasanton, CA, USA) for 30 min at room temperature. All reactions were developed using a diaminobenzidine chromogen solution (kit REVEAL-Biotin-Free Polyvalent DAB; Spring Bioscience, Inc.). The counterstaining was performed with Harris hematoxylin. The slides were dehydrated, cleared and mounted. Positive and negative control were used for each primary antibody according to the manufacturer recommendation. For all antibodies, positive staining was defined as a brown stain in the cell membrane. Expression was evaluated by calculating a total immunostaining score (TIS) as the product of a proportion score (PS) and an intensity score (IS). The PS describes the estimated fraction of positively stained tumor cells (0, none; 1, <10%; 2, 10-50%; 3, 51-80%; 4, >80%). The IS represents the estimated staining intensity as compared with control (0, no staining; 1, weak; 2, moderate; 3, strong). The TIS (TIS=PSxIS) ranges from 0 to 12 with only nine possible values (that is, 0, 1, 2, 3, 4, 6, 8, 9 and 12). Four subgroups were defined: No expression, TIS 0; weak expression, TIS 1-4; moderate expression, TIS 6, 8; intense expression, TIS 9,12. EpCAM 'overexpression' has been defined previously as a TIS>4 (13).

Results

Claudin 4. Overexpression of Claudin 4 was observed in all samples, including adenocarcinoma samples from different primary sites and carcinomas of different histological types such as squamous cell carcinoma (cervical), small cell carcinoma (lung), and urothelial carcinoma (bladder). The TIS values ranged from 6-12 corresponding to moderate and intense expression (Table II; Figs. 1 and 2).

Moc-31. EpCAM overexpression as detected by Moc-31 antibody was observed in 96.36% (53/55) of all carcinoma samples. Overexpression was found in all adenocarcinoma samples, from all primary sites with TIS values ranging from 6-12 corresponding to moderate and intense expression. Among other histological types, overexpression was observed in small cell carcinoma (lung). The expression was weak in squamous cell carcinoma (cervical) and urothelial carcinoma (bladder). (Table II; Figs. 1 and 2).

Ber-EP4. EpCAM overexpression as detected by Ber-EP4 was observed in 90.90% (50/55) of all carcinoma samples. Overexpression was found in almost all adenocarcinoma samples from all primary sites, except in two samples of breast. The overexpression in adenocarcinomas of the breast was observed in 80% of the samples. The TIS values in samples with overexpression ranged from 6-12, corresponding to moderate and intense expression. No overexpression was observed in all non-adenocarcinoma histological types of carcinoma analyzed: Small cell carcinoma (lung), squamous cell carcinoma (cervical) and urothelial carcinoma (bladder). (Table II; Figs. 1 and 2).

158210. EpCAM overexpression as detected by clone 158210 was observed in 90.90% (50/55) of all carcinoma samples.

Table I. Primary antibodies.

Target molecule	Manufacturer	Clone	Dilution	Control
EpCAM	R&D Systems	158210	1:1,200	Gastric cancer
Epithelial related antigen	DAKO	Moc-31	1:200	Gastric cancer
Epithelial related antigen	DAKO	Ber-EP4	1:300	Breast
Claudin-4	NOVEX	3E2C1	1:200	Gastric cancer

Table II. Overexpression of Claudin-4 and EpCAM (as detected by Moc-31, Ber-EP4 and 158210) according to primary sites of carcinoma and histological types.

	Claudin-4 Overexpression n (TIS range)		Moc-31 Overexpression n (TIS range)		Ber-EP4 Overexpression n (TIS range)		158210 Overexpression n (TIS range)	
Carcinoma	Absence	Presence	Absence	Presence	Absence	Presence	Absence	Presence
Adenocarcinomas								
Lung (n=15)	0	15 (6-12)	0	15 (6-12)	0	15 (6-12)	0	15 (6-12)
Ovary (n=12)	0	12 (6,12)	0	12 (6,12)	0	12 (12)	2 (4)	10 (6,12)
Breast (n=10)	0	10 (6-12)	0	10 (6-12)	2 (4)	8 (6-12)	1 (4)	9 (6-12)
Stomach (n=4)	0	4 (6-12)	0	4 (6-12)	0	4 (6-12)	1 (4)	3 (6-12)
Colon (n=2)	0	2 (8,12)	0	2 (8,12)	0	2 (8,12)	0	2 (6,12)
Cervix (n=2)	0	2 (8,12)	0	2 (8,12)	0	2 (8)	0	2 (8)
Biliary tract (n=3)	0	3 (8,12)	0	3 (8,12)	0	3 (6-12)	0	3 (8,12)
Pancreas (n=1)	0	1 (12)	0	1 (12)	0	1 (6)	0	1 (6)
Endometrium (n=1)	0	1 (12)	0	1 (12)	0	1 (6)	0	1 (6)
Unknown site (n=2)	0	2 (8,12)	0	2 (12)	0	2 (12)	0	2 (6,12)
Subtotal Adenocarcinomas (n=52)	0	52 (6-12)	0	52 (6-12)	2 (4)	50 (6-12)	4 (4)	48 (6-12)
Other histological types								
Squamous carcinoma (cervix) (n=1)	0	1 (9)	1 (1)	0	1 (2)	0	0	1 (9)
Urothelial carcinoma (bladder) (n=1)	0	1 (12)	1 (4)	0	1 (2)	0	1(2)	0
Small cell carcinoma (lung) (n=1)	0	1 (8)	0	1 (12)	1 (4)	0	0	1 (6)
Total (n=55)	0	55	2	53	5	50	5	50

TIS, Total immunostaining score.

Among adenocarcinoma samples, overexpression was detected in almost all samples, except in some from ovary, breast, and colon. The overexpression in adenocarcinomas of the ovary, breast and stomach was observed in 83.33, 90 and 75% of the samples, respectively. The TIS values in samples with overexpression ranged from 6-12, corresponding to moderate and intense expression. Among different histological types analyzed, overexpression was observed in small cell carcinoma (lung) and squamous cell carcinoma (cervical). The expression was weak in urothelial carcinoma (bladder). (Table II; Figs. 1 and 2).

Discussion

The absence of EpCAM expression in normal cells found in the cavities lining and fluids (mesothelial cells, leukocyte and macrophages) would indicate that EpCAM is a highly specific marker for diagnosis and target therapy for carcinomas in effusions. There are several immunocytochemical markers for identification of non-neoplastic cells found in the effusions; calretinin and HBME, for instance, are routinely used for mesothelial cells and CD68 for macrophages, respectively (14).

In the present study, the expression of EpCAM was evaluated in metastatic carcinomas of effusions originated from different primary sites and of distinct histological types. We compared the immunoreactivity of antibodies that react to different epitopes of the extracellular domain of EpCAM. We used two antibodies against epitopes in the EGF-like domain I (clones Moc-31 and Ber-EP4) and one antibody against the epitope in the cysteine-poor region (158210) of EpCAM, all of them commercially available. EpCAM expression was evaluated by calculating the total immunostaining score (TIS), which is the product of the proportion score and the intensity score. This score has been used to evaluate the expression

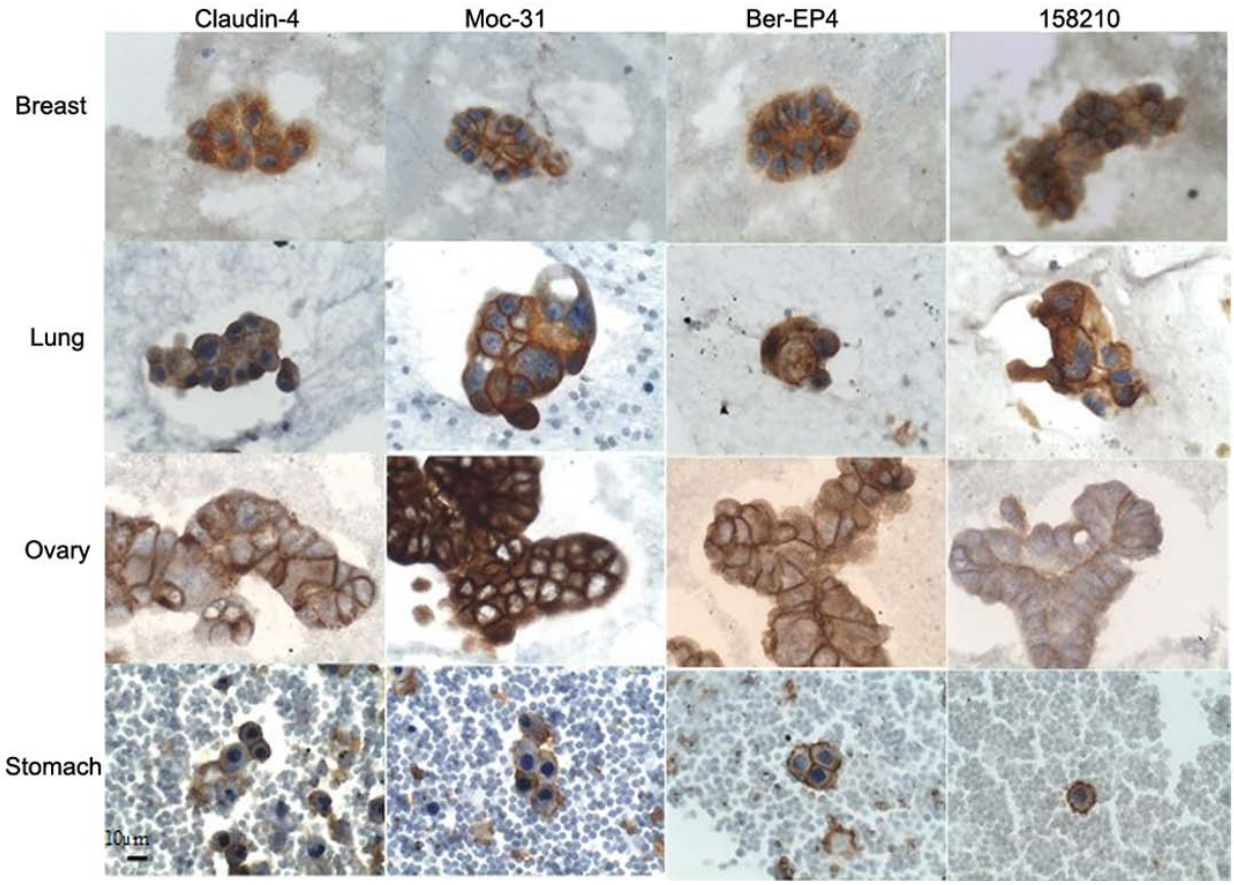

Figure 1. Immunocytochemistry showing overexpression in adenocarcinoma of breast, lung, ovary and stomach from effusion using anti-Caudin-4 and three clones of anti-EpCAM antibodies (MOC-31, Ber-EP4 and 158210). Magnification, x400. EpCAM, epithelial cell adhesion molecule.

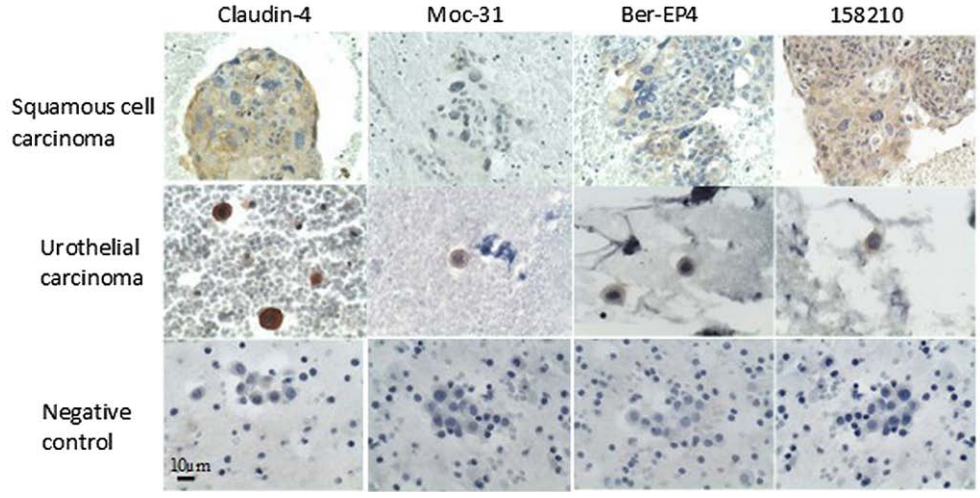

Figure 2. Immunocytochemistry of effusion samples (magnification, x400). Overexpression of Claudin-4 in metastatic squamous cell carcinoma of cervix and in metastatic urothelial carcinoma of bladder. By using Moc-31 and Ber-EP4 no EpCAM overexpression was detected in metastatic squamous cell carcinoma of cervix or in metastatic urothelial carcinoma of bladder. By using clone 158210, EpCAM overexpression was detected in metastatic squamous carcinoma of cervix but not in metastatic urothelial carcinoma of bladder. In the negative control (sample without carcinoma/patient with heart failure), no expression of Claudin-4 and EpCAM in mesothelial and macrophages was detected. EpCam, epithelial cell adhesion molecule.

of EpCAM in surgical histological specimens with primary carcinoma. Here, for the first time, we applied this score in cytological effusion samples to make the expression in effusions and that in primary sites, previously established by other studies, comparable.

All immunocytochemistry reactions were performed on cell block sections which provide a better morphologic interpretation with less background staining when compared to Cytospin and ThinPrep samples (15). The results most closely approximated those reported in the surgical pathology specimens. The

cell block preparation method used was plasma/tromboplastin, which in comparison with other methods, is easily prepared and produce the best cell block results in regards to cellularity, cell distribution and background on immunocytochemistry (16).

Claudin-4 has been described as the most sensitive marker to distinguish adenocarcinomas from reactive and malignant mesothelial cells in cytology of effusions, so the results of reactions with anti-claudin-4 were used as a reference for comparison with the results with anti-EpCAM antibodies (17,18). In the present study, Claudin-4 overexpression was observed in all adenocarcinoma samples and its TIS values was higher than those obtained with anti-EpCAM antibodies. Anti-claudin-4 was also superior to anti-EpCAM antibodies for the detection of other types of carcinomas, such as squamous cell and urothelial carcinoma.

Independently of the clone used, EpCAM overexpression was observed in almost all adenocarcinoma samples. However, different degrees of EpCAM expression were observed depending on the site of origin and histological type of carcinoma and depending on the antibody used. Heterogeneous detection of EpCAM was mainly observed in types of carcinoma other than adenocarcinoma.

Given that both (clones Moc-31 and Ber-EP4) antibodies react with epitopes in the same extracellular domain of EpCAM, one would expect similar reactivity with these antibodies. However, Balzar *et al* (19) suggested that different conformational states of the cell surface EpCAM protein might hide some epitopes leading to subpopulations of EpCAM and thus heterogeneous affinity. In present study, Moc-31 presented higher TIS values for adenocarcinomas but a lower TIS value for squamous cell carcinoma in comparison with Ber-EP4. For adenocarcinoma of origin in breast, EpCAM overexpression was observed in 80% of samples by using Ber-Ep4 in comparison to 100% EpCAM overexpression with Moc-31.

Similarly to present results on metastatic carcinoma, in surgical specimens with primary carcinomas, different degrees of EpCAM expression has also been observed according to site of origin and histological type of carcinoma (20-22). Overall, the percentage of positive samples and TIS values for EpCAM were higher in our metastatic carcinoma samples than in the primary carcinoma samples analyzed in previous studies (20-22). In the case of breast cancer, our TIS values for EpCAM were higher than those obtained in previous studies in primary and metastatic carcinomas for lymph node and CNS (20).

The weak EpCAM expression in urothelial and squamous cell carcinoma observed in present study is in accordance with the results of studies in primary carcinoma samples (21). This result indicates that if EpCAM specific antibodies are intended to be used for treatment in patients with these histological types of cancer, prior immunohistochemical evaluation of EpCAM expression should be recommended.

To our knowledge, for the first time, EpCAM expression was evaluated in metastatic carcinoma from effusion by using an antibody directed against an epitope in the cysteine poor region of the ectodomain of the EpCAM molecule. By using 158210, overexpression was observed in 90.90% of all carcinoma samples. With regard to adenocarcinoma samples, almost all primary sites showed overexpression in all samples, except some samples of ovary, breast, and colon. Among the antibodies, it was the only one that detects overexpression in the sample of squamous cell carcinoma.

In healthy adult tissue, EpCAM is expressed in cell membrane of simple, pseudo-stratified, and transitional epithelia, but no expression can be detected in the differentiated cells of normal squamous stratified epithelia. In primary squamous cell carcinoma (SCC) of the uterine cervix, EpCAM expression have showed heterogeneity depending on the antibody clone (20-22). Similarly, in metastatic samples of effusion, other authors showed that EpCAM expression in SCC was lower than in adenocarcinoma samples, 67% vs. 100%, respectively (23). In a previous study, anti-EpCAM monoclonal antibodies that recognize the 6 kDa fragment (located distant from the cell membrane and removed after cleavage at the position Arginine80/Arginine81) and the 32 kDa fragment (located proximal to the membrane) of EpCAM extracellular domain were generated and used to compare detection of EpCAM expression in cervical SCC (24). These authors showed that EpCAM expression is consistently detected on SCC of cervix by using anti-EpCAM that recognizes the membrane-proximal part (24). The clone 158210 used in present study detects an epitope found in the extracellular domain between amino acids 136 and 265 and, therefore, located at the membrane-proximal part of the extracellular domain. Thus, the high TIS value of EpCAM expression in squamous cell carcinoma by using the clone 158210 is in agreement with the results of this previous study and suggest a potential use of antibodies directed against an epitope in the cysteine poor region of the ectodomain of the EpCAM molecule for detection of this type of carcinoma in effusion.

Another commercially available cysteine-poor region-specific EpCAM antibody is 311-1K1. In a previous study, this antibody and Ber-Ep4 were used to evaluate EpCAM expression in tissue sections of colorectal carcinoma (25). These authors showed that in contrast to the tumor mass, budding cells of colorectal carcinoma displayed lack of membranous but highly increased cytoplasmic EpCAM staining. Significant cytoplasmic EpCAM staining was also observed in the present study by using all three EpCAM antibodies and anti-Claudin-4.

EpCAM expression defined by IHC predicts whether patients may benefit with a specific EpCAM targeting agent and its possible therapy response. First studies targeting EpCAM lacked patient randomization according to the actual EpCAM status on tumor cells and this can explain the disparate results sometimes obtained (26).

The main limitation of the present study was the small number of samples from some sites of origin of adenocarcinoma and of different histological types of carcinoma. However, even with this small sampling, it was possible to demonstrate heterogeneity in the EpCAM expression by using antibodies against different epitopes of its ectodomain.

Overall, most samples of metastatic carcinoma from effusions showed overexpression of EpCAM. However, there are significant variations in its expression according to the primary site and histological type of the carcinoma and depending on the antibody used. Thus, the use of more than one type of anti-EpCAM would increase the chance of its detection in metastatic carcinoma of effusion.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Federal District Research Support Foundation (FAP-DF) and the Foundation for Teaching and Research in Health Sciences (FEPECS), FAHUB, CAPES, CNPQ.

Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

TKSB and MIMJ made substantial contributions to the conception and design of the study. MVC acquired clinical data. VMF and ABM made substantial contributions to the design of the experiments and the interpretation of the results obtained with different antibodies. TMMLC, NVH and IAO performed immunocytochemistry, including the optimization of the experimental conditions. LMRB, LLF and RVMS acquired samples and prepared microscopy slides. DLMV and ACS performed cell blocks. GCC and AMP analyzed the clinical data. FPC, IP and LMSM performed microscopy examinations. LMSV and GHST analyzed and interpreted sample data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics in Research Committee of Brasilia University, Brazil. Informed consent was obtained from all individual participants included in the study. CAAE: 37194114.4.0000.5553.

Patient consent for publication

Informed consent was obtained from all individual participants included in the study. CAAE: 37194114.4.0000.5553.

Competing interests

The authors declare that they have no competing interests.

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