A panel of markers for identification of malignant and non-malignant cells in culture from effusions

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Abstract. The aim of the present study was to identify cell types in primary culture from malignant and non-malignant effusions. Effusion samples were subjected to cytology and culture. Immunocytochemistry was performed in cytological slides to evaluate malignancy (positivity for malignancy markers) and in culture slides for identification of cell types in growth. A total of 143 effusion samples (pleural n=76; peritoneal n=37; pericardial n=4; and peritoneal lavage n=26) were analyzed. Cell growth was observed in 34.9% of all samples and immunocytochemistry for identification of cell types in culture slides was conclusive in 90% of them. In non-malignant samples (n=28), growth of mesothelial cells, macrophages and of both cell types was identified in 82.14, 10.71 and 7.14%, respectively. In malignant samples (n=17, all carcinomas), growth of malignant epithelial cells and of both malignant epithelial and mesothelial cells was identified in 41.17 and 23.52%, respectively. In the remaining 35.29% of malignant samples, the only cells in growth were mesothelial and/or macrophages instead of malignant epithelial cells. In conclusion, in culture of malignant effusions, mesothelial cells may be simultaneously identified with malignant epithelial cells. Besides, mesothelial cells and macrophages may be the only cells identified in malignant effusion culture. Therefore, a broad panel of cell markers should be used for unmistakable identification of cells in studies of effusion primary culture. The ideal malignant effusion sample to obtain culture of neoplastic cells should be that without the presence of mesothelial cells and macrophages.

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Introduction

Malignant effusion cell culture of patients with different cancers have been used to assess the response to chemotherapeutic agents, to provide *in vitro* characterization of neoplastic cell lines, to investigate tumoral heterogeneity and to understand the role of epithelial-mesenchymal transition (EMT) and cancer stem cells (CSCs) in metastasis and in high therapeutic failure rates (1-8).

Cytology of effusions (pleural, peritoneal and pericardial fluid) and peritoneal lavage is a routine procedure in pathological anatomy laboratories for the diagnosis, staging, determining the primary site of metastatic cancer and follow-up of patients with high incidence and mortality cancers such as carcinoma of breast, lung, ovary and stomach (9-11). The cell types usually identified by cytology of benign effusion are mesothelial cells, macrophages and leukocytes. Besides these cells, neoplastic cells may also be observed in malignant effusions (9-11). Effusions with high cellularity and characteristic morphological aspects enable to define the nature of the malignant cells (9-11). However, it should be noted that there are instances when the distinction between metastatic cancer cells and reactive or neoplastic mesothelium can be challenging (12-17). In these cases, immunocytochemistry may elucidate the origin of the atypical cells (12-17). Immunocytochemical panels typically include markers of mesothelial and of malignant epithelial cells but these markers do not exhibit high specificity and sensitivity and a broad panel of immunocytochemical markers is used for differentiating these cells (12-17).

Identification of cell types in culture of effusions is even more difficult than in cytology because the growing cells frequently undergo morphological and functional changes that in turn may also result in different expression pattern of immunocytochemical markers. In addition, there is the possibility of

Table I. Antibody used in immunocytochemistry.

Antibody	Source	Clone	Dilution
Anti-epithelial related antigen	Dako	MOC-31	1:100
Anti-IMP3	Dako	69.1	1:500
Anti-Claudin 4	Abcam	AB15104	1:200
Anti-Calretinin	Dako	DAK-Calret 1	1:50
Anti-human mesothelial cell	Cell Marque	HBME-1	1:50
Anti-Wilms' tumor 1 (WT-1)	Dako	6F-H2	1:300
Anti-CD68	BioCare Medical	KP1	1:100
Anti-vimentin	Dako	Clone V9	1:100
Anti-pan-cytokeratin (PANCK)	Dako	AE1/AE3	1:50
Anti-LCA	Dako	4KB5	1:50
Anti-Melan-A	Dako	A103	1:25

growth of various cell types (mesothelial, macrophages and epithelial malignant) simultaneously and failure to correctly identify them can interfere with subsequent research results. Therefore, in view of the potential use of culture of effusions and the need for the correct identification of cell types grown in culture, the aim of the present study was to identify by immunocytochemistry cells in culture from malignant and non-malignant effusions.

Materials and methods

Samples. Samples of effusions (pleural, peritoneal and pericardial) and peritoneal lavage were obtained at the Department of Pathology of Brasilia University Hospital, Brazil, between 2012 to 2015. The study protocol was approved by the Human Ethics Review Committee of the Brasilia University.

Cytological and immunocytochemical analyses. All samples were centrifuged and the sediment was fixed in alcohol and stained by the Papanicolaou method. Cytology was considered positive for malignancy, negative, suspicious or unsatisfactory. Immunocytochemistry was performed in cytological slides to evaluate malignancy: the samples were divided into malignant (with positivity malignancy markers) and non-malignant (lack of positivity for malignancy markers).

Cell culture. The initial volume of the samples ranged from 10 to 50 ml. The unfixed samples were centrifuged, the supernatant was removed, and the cells were resuspended in 500 μl of medium and then seeded onto a 4-well chamber slide, containing equal amount of complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% bovine fetal serum (FBS) and antibiotics and antifungals 2% (penicillin, streptomycin and amphotericin B, 200 U/ml, 200 mg/ml and 2.5 μg/ml). Samples were seeded in duplicate and culture slides were incubated at 37˚C and 5% CO_2 for a total of 7 days, with medium change on the third day. On days 2 and 7, samples were washed and fixed in alcohol. Immunocytochemistry was also performed in culture slides for identification of cell types in growth.

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. After washing with PBS, the slides were incubated with a secondary antibody for 30 min at room temperature and subsequently with the streptavidin-biotin-peroxidase complex (LSAB+; DAKO A/S, Glostrup, Denmark; K-690) for 30 min at room temperature. All reactions were developed using a diaminobenzidine chromogen solution (Dab substrate chromogen system-K3468; Dako). 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's recommendations. Positive staining was defined as a strong brown stain in more than 1% of cells in the cytoplasm (Pan-cytokeratin, CD68, vimentin and melan), membrane (HBME and LCA), nucleus (WT1), cytoplasm and membrane (MOC-31 and Claudin 4), cytoplasm and nucleus (calretinin and IMP3). The expression of at least two of the following markers was considered for identification of the mesothelial cell: calretinin, HBME and WT1. The expression of at least two of the following markers was considered for identification of the malignant epithelial cells: MOC, IMP3 and Claudin 4. The identification of macrophages was performed using the marker CD68 and lack of expression of epithelial markers. LCA and HMB-45 were used for identification of lymphoma and melanoma, respectively.

Results

Samples. A total of 143 samples (pleural effusion n=76; peritoneal effusion n=37; pericardial effusion n=4; and peritoneal lavagen n=26) were analyzed. In 32.86% (47/143) of samples, patients presented with current or previous histological diagnostic of cancer (carcinoma n=45; lymphoma n=1; and melanoma n=1).

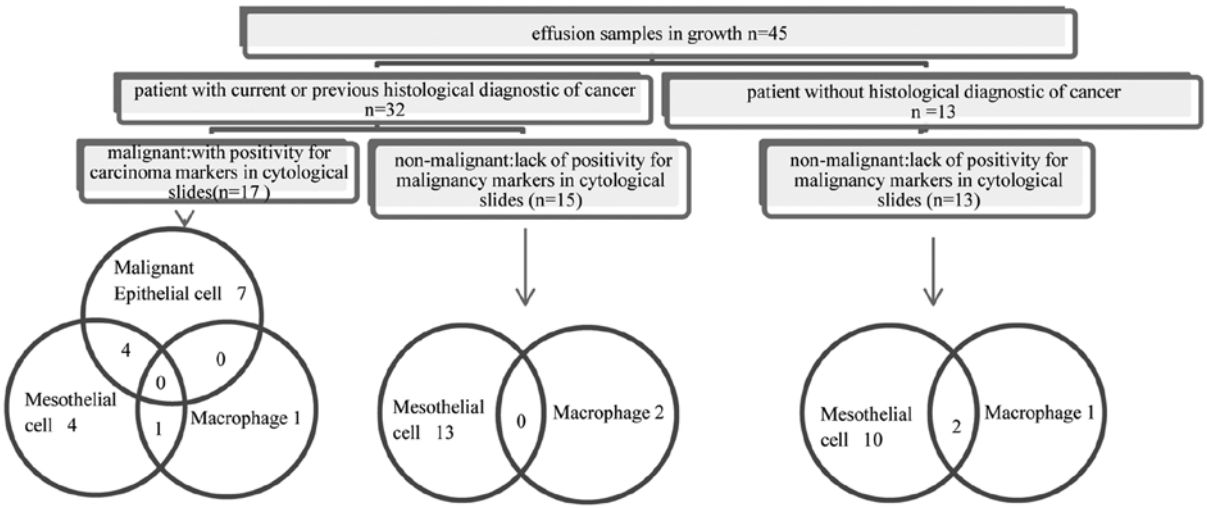

Figure 1. Cell types identified by immunocytochemistry in culture slides of malignant and non-malignant effusions.

Cytology, culture and immunocytochemistry. Cell growth was observed in 34.96% (50/143) of all samples (pleural effusion n=31 and peritoneal effusion n=19). After two days in culture, adherent cells formed a flat monolayer, and appeared homogenous and polygonal in shape on the seventh day. Because the cells were similar morphologically, immunocytochemistry was performed to identify the different cellular types in culture.

The identification of cell types in culture by immunocytochemistry was conclusive only in 90% (45/50) of samples that showed growth. In the remaining 5 samples, low cellularity prevented the use of all necessary markers to complete immunocytochemistry analysis. In 71.11% (32/45) of samples in which immunocytochemistry was conclusive, patients presented with current or previous histological diagnostic of cancer but only in 37.77% (17/45), the samples were malignant (with positivity for carcinoma markers). In 62.22% (28/45), the samples were non-malignant (lack of positivity for malignancy markers) (Fig. 1).

The types of cells in culture identified by immunocytochemistry were mesothelial cell, malignant epithelial cell and macrophages.

In non-malignant samples, growth of mesothelial cells, macrophages and of both cell types were identified in 82.14% (23/28), 10.71% (3/28) and 7.14% (2/28), respectively. In malignant samples, growth of malignant epithelial cells and of both malignant epithelial and mesothelial cells was identified in 41.17% (7/17) and 23.52% (4/17), respectively. In the remaining 35.29% (6/17) of malignant samples, the only cells in growth were mesothelial and/or macrophages instead of malignant epithelial cells (Fig. 1).

Presence of mesothelial cells in culture was identified by positivity for at least two of the markers calretinin, WT1 or HBME. The pattern of expression of the markers in adherent mesothelial cells in culture was similar to that of adjacent non-adherent mesothelial cells and to that of cells in cytology; cytoplasm and nucleus for calretinin, membrane for HBME and nucleus for WT1 (Figs. 2 and 3). Expression of non-specific markers was also observed in the adherent mesothelial cells: pan-cytokeratin and vimentin. Malignant epithelial cells in culture were identified by positivity for at least two of the following markers: MOC-31, Claudin 4 and IMP3. The pattern of expression of the markers in adherent malignant epithelial cells in culture was similar to that of adjacent non-adherent malignant epithelial cells and to that of cells in cytology: cytoplasm and membrane for MOC-31 and Claudin 4, cytoplasm and nucleus for IMP3 (Fig. 3). Macrophages were identified by positivity for CD68 and lack of expression of epithelial markers. The staining pattern (cytoplasm) in adherent cells was similar to that of non-adherent adjacent cells and to that of cells in cytology.

Discussion

The aim of the present study was to identify, by a panel of immunocytochemical markers, cell types in primary culture from effusions. Although a large number of samples have been subjected to culture in this study, growth was obtained in only 34% of them. The low cellularity, mainly in peritoneal lavage, may explain, at least partially, the low number of samples with growth. To minimize the loss of cells, culture was performed directly on chamber slides and the interval between sample collection and culture of the samples was lesser than 48 h, a period when the samples were kept at 4˚C until culture. After culture and cytological analyses, the remainder of the sample was kept at 4˚C for up to 2 weeks. Notably, in some of these samples, the presence of viable and capable of growth mesothelial cells was noted after such long-period storage (results not shown). Lack of adherence of the cells was another limiting factor to obtain a higher number of cultured samples. But cellularity and time of culture does not seem to be a determinant of adherence and growth of malignant epithelial cells in culture because in some samples in which many malignant epithelial cells (isolated or grouped) were present, no cell growth or only growth of mesothelial cells was identified, despite the high cellularity of malignant epithelial cells and even after 7 days of culture. This is in accordance with recent results which showed that cancer stem cells or tumor-initiating cells tend to grow in three dimensional cultures (18).

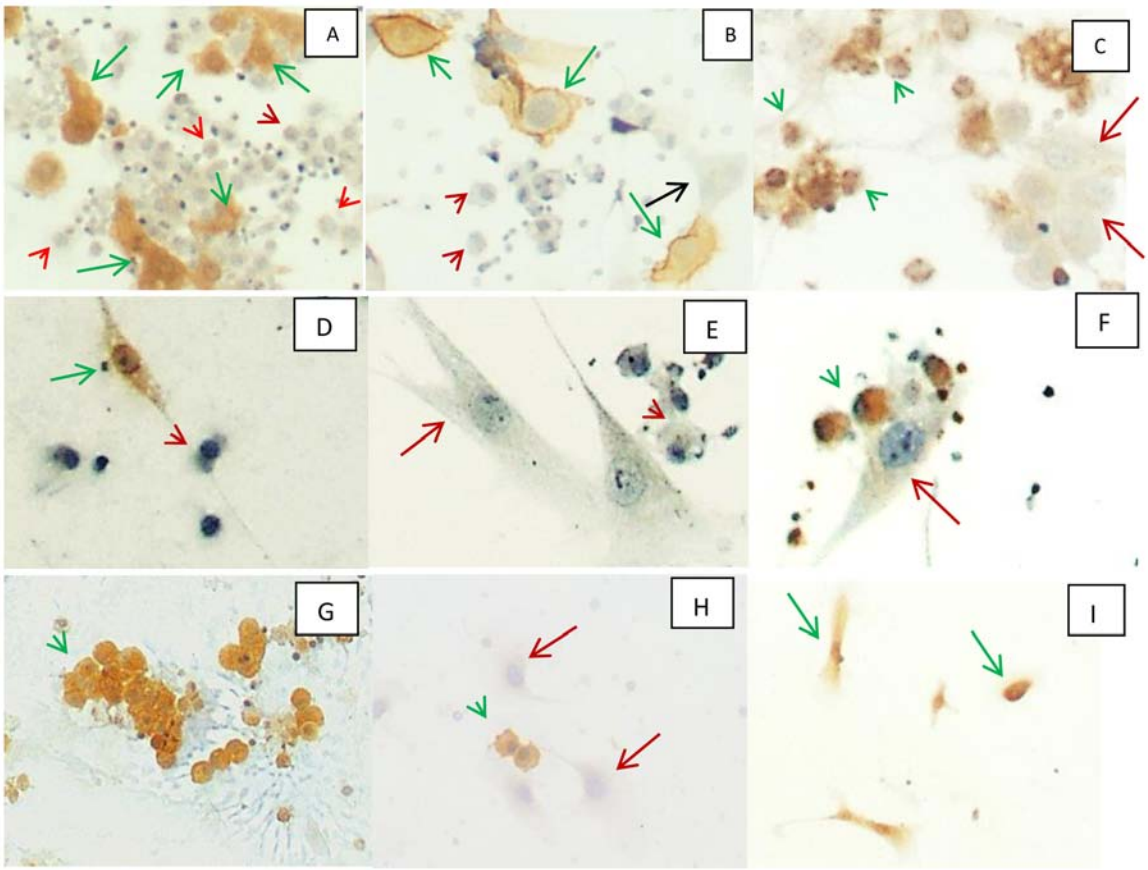

Figure 2. (A-C), culture of mesothelial cells from pleural effusion of patient with history of pneumonia. (A) Immunocytochemistry for calretinin (magnification, x100). Positive in cytoplasm and nucleus of adherent mesothelial cell (green arrow), negative in non-adherent macrophages (red arrowhead). (B) Immunocytochemistry for HBME (magnification, x100). Positive (green arrow) and negative (black arrow) in membrane of adherent mesothelial cells and negative in non-adherent macrophages (red arrowhead). (C) Immunocytochemistry for CD68 (magnification, x100). Positive in cytoplasm of non-adherent macrophages (green arrowhead), negative in adherent mesothelial cells (red arrow). (D-F) Culture of mesothelial cells from pleural effusion of patient with history of breast carcinoma and lack of positivity for carcinoma markers in cytological slides. (D) Immunocytochemistry for calretinin (magnification, x200). Positive in cytoplasm and nucleus of adherent mesothelial cells (green arrow), negative in non-adherent macrophages (red arrowhead). (E) Immunocytochemistry for MOC-31 (magnification, x200). Negative in adherent mesothelial cells (red arrow) and negative in non-adherent macrophages (red arrowhead). (F) Immunocytochemistry for CD68 (magnification, x200). Positive in cytoplasm of non-adherent macrophages (green arrowhead), negative in adherent mesothelial cells (red arrowhead). (G-I) culture of mesothelial cells from pleural effusion of patient with history of breast carcinoma and positivity for carcinoma markers in cytological slides. (G and H) Immunocytochemistry for MOC-31 (magnification, x100). Positive in cytoplasm and membrane of non-adherent epithelial malignant cells (green arrowhead) and negative in adherent mesothelial cell (red arrow). (I) immunocytochemistry for calretinin (magnification, x100). Positive in cytoplasm and nucleus of adherent mesothelial cells (green arrow).

Morphologically, adherent mesothelial and malignant epithelial cells were indistinguishable in appearance and size, whereas macrophages were proportionally smaller as compared with those cells.

Using immunocytochemistry, it was possible to identify cell types in 90% (45/50) of the samples in culture. The expression pattern (nucleus, membrane and/or cytoplasm) of the markers remained at 2 and 7 days of growth, but one limitation of the present study was lack of analysis of markers expression for longer periods of time.

The predominant cell type of effusions in culture was mesothelial cells and they were present mainly in inflammatory benign or malignant effusions. Mesothelial cells are specialized epithelial cells that line the serous cavities (pleural, pericardial and peritoneal) (19). Under normal homeostasis, mesothelial cells exhibit limited cell proliferation, with only 0.16-0.5% of cells within the mesothelium undergoing mitosis at any one time (19,20). The rate of mitosis increases to 30-60% following injury to the mesothelium, and this is attributed in part to increased levels of growth factors and cytokines (20-22). Culture and isolation of mesothelial cells has been used in studies on the role of mesothelial cells in the progression of cancer in malignant effusion (23-25).

A major contribution of this study was to demonstrate that in 65.62% of effusions of patient with current or previous histological diagnosis of cancer and in 35.29% of malignant effusion, the only cells found in culture were mesothelial and/or macrophages instead of malignant epithelial cells. Besides this, in 23.52% of malignant effusion, mesothelial and malignant epithelial cells were growing simultaneously in culture. When the objective is to obtain primary culture of malignant epithelial cells from a malignant effusion, the ideal sample should be without the presence of mesothelial cells and macrophages which are cells generally present when there is associated inflammation.

As the mesothelial cells were morphologically indistinguishable from malignant epithelial cells and no single marker is characterized by 100% specificity and sensitivity for

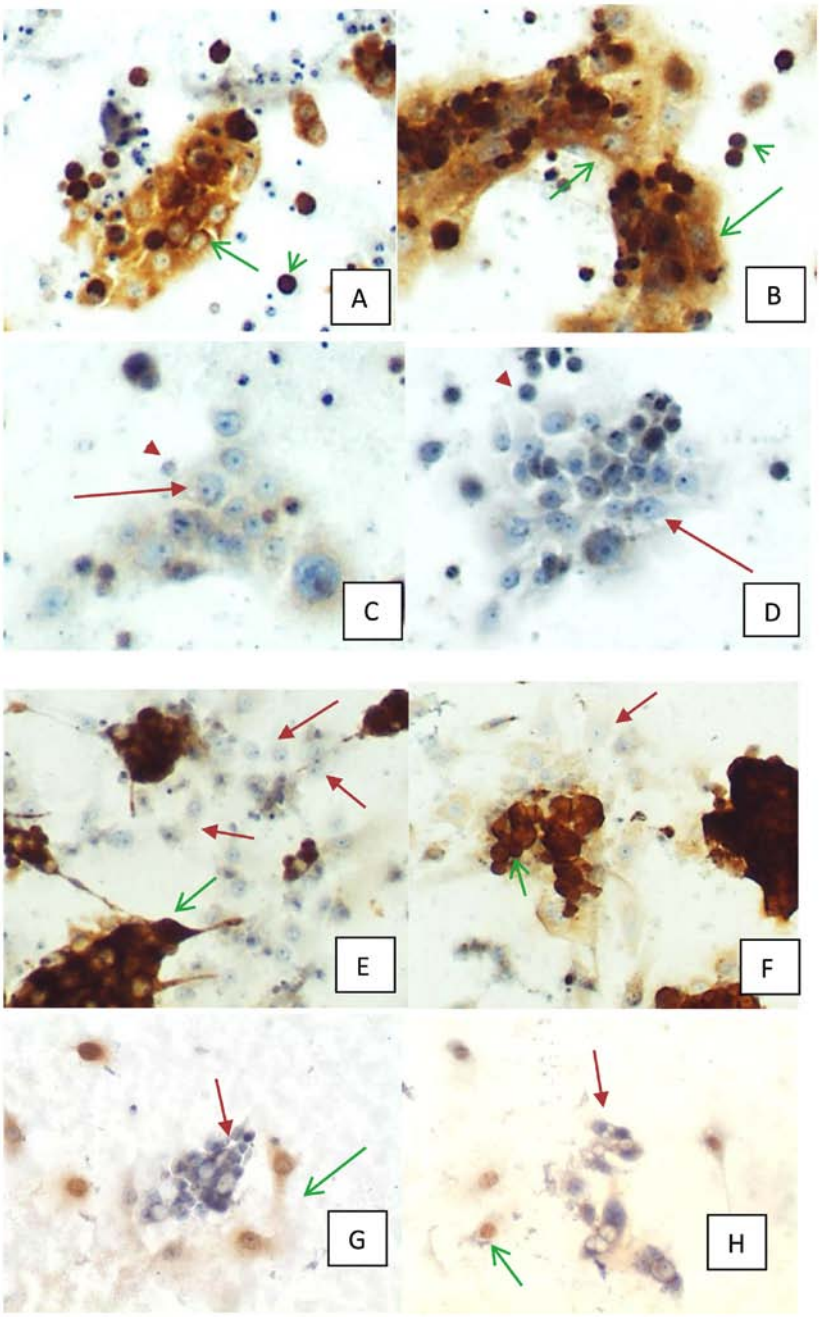

Figure 3. (A-D) culture of malignant epithelial cells from pleural effusion of a patient with history of esophagic cancer and positivity for carcinoma markers in cytological slides. (A) Immunocytochemistry for MOC-31 (magnification, x100). Positive in membrane and cytoplasm of adherent (green arrow) and non-adherent (green arrowhead) malignant epithelial cells. (B) Immunocytochemistry for IMP3 (magnification, x100). Positive in cytoplasm of adherent (green arrow) and non-adherent malignant epithelial cells (green arrowhead). (C) Immunocytochemistry for calretinin (magnification, x100). Negative in adherent (red arrow) and non-adherent (red arrowhead) malignant epithelial cells. (D) Immunocytochemistry for HBME (magnification, x100). Negative in adherent (red arrow) and non-adherent (red arrowhead) malignant epithelial cells. (E-H) Culture of both malignant epithelial and mesothelial cells from pleural effusion of patient with history of gastric carcinoma and positivity for carcinoma markers in cytological slides. (E) Immunocytochemistry for IMP3 (magnification, x100). Positive in cytoplasm of adherent malignant epithelial cells (green arrow), negative in adherent mesothelial cells (red arrow). (F) Immunocytochemistry for MOC-31 (magnification, x100). Positive in cytoplasm and membrane of adherent malignant epithelial cells (green arrow), negative in adherent mesothelial cells (red arrow). (G) Immunocytochemistry for calretinin (magnification, x100). Positive in cytoplasm and nucleus of adherent mesothelial cells (green arrow), negative in adherent malignant epithelial cells (red arrow). (H) Immunocytochemistry for WT1 (magnification, x100). Positive in nucleus of adherent mesothelial cells (green arrow), negative in adherent malignant epithelial cells (red arrow).

distinguishing these cells, a broad panel of markers should be used for the growth of mesothelial cells and macrophages not to be confused with growth of neoplastic cells in malignant effusions culture.

To the best of our knowledge, we have for the first time used a broad painel of immunocytochemical markers for identification of cells in primary culture from malignant and non-malignant effusions culture. The markers used here to identify mesothelial cells in growth were calretinin, HBME and WT1, but alternative markers could have be used such as thrombomodulin (14). Calretinin is a high sensitivity and specificity marker and widely used (14,16,26,27). HBME was used, in the present study, for

identification of mesothelial cells but it has a low sensitivity when compared to calretinin. Moreover, HBME can be expressed in certain types of carcinoma, such as lung, ovarian, breast, colon and stomach carcinoma (14,28). WT1 expression can be detected in benign and malignant mesothelial cells. However, WT1 is a marker that is expressed in most of primary ovarian carcinomas and has been used to distinguish carcinoma of ovarian origin from carcinoma of other primary sites. Thus, although the expression of HBME and WT-1 could potentially cause some difficulty in the correct interpretation of effusion specimens, concomitant usage of other mesothelial cells and malignant epithelial cell markers could prove helpful (15,29).

The markers used to identify malignant epithelial cells were MOC-31, Claudin 4 and IMP3, but several other adenocarcinomas markers have been used to distinguish malignant epithelial cells from mesothelial cells such as B72.3, CEA and Ber-EP4 (30-34). Besides, some primary site markers of adenocarcinoma can also contribute to distinction between mesothelial and epithelial malignant cells such as estrogen receptor, progesterone receptor, GATA3, mammoglobin, GCPD15 for breast; Napsin-A and TTF-1 for lung; CDX-2, villin, SATB2 for gastrointestinal tract; PSA for prostate; PAX8, HBME and estrogen receptor for ovary; CD10 and PAX 8 for kidney; hepatocyte-specific antigen for liver. As no single marker presents 100% specificity and sensitivity, positivity for a combination of markers have been used for identification of primary site markers of adenocarcinomas (35-45). For the diagnosis of squamous cell carcinoma, the negativity for MOC-31, negativity for mesothelial cell markers, and positivity for P63 may be useful (46).

In conclusion, the present study showed that in culture of malignant effusions, mesothelial cells may be simultaneously identified with malignant epithelial cell. Mesothelial cells and macrophages may be the only cells identified in malignant effusion culture. Therefore, a broad panel of cell markers should be used for identification of cells in studies of effusion primary culture. The ideal malignant effusion sample to obtain culture of neoplastic cells should be that without the presence of mesothelial cells and macrophages. These data will prevent future errors in studies using cells isolated from effusion cultures.

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