Artigo submetido em 21/11/07; aceito para publicação em 17/4/08

Utility of AgNOR, Immunocytochemistry for CEA and Tumor Markers for Diagnosis in Serous Fluids

Utilidade da AgNOR, Imunocitoquímica para CEA e Marcadores Tumorais no Diagnóstico, utilizando Fluidos Serosos

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Abstract

Objective: The evaluation of serous fluids stained by morphological methods lacks, in many cases, the necessary accuracy to obtain the correct diagnostics. The objective of this work was to establish the value of complementary tools for the improvement of diagnosis in serous effusions. **Methods:** Fifty-six serous effusions were processed for morphological staining, immunocytochemistry of carcinoembryonic antigen (CEA), AgNOR counting and electrochemiluminescense immunoassay for tumor markers (TM): CEA, Ca125 and CYFRA 21-1. TM assays were also performed in sera from the same patients. The Sensitivity (Se) and Specificity (Sp) were evaluated for all the methods. **Results:** *Cytology:* Se 73%, Sp 100%, *CEA by immunocytochemistry:* Se 96%, Sp 75%, *AgNOR:* Se 86%, Sp100%, *TM:* a) in fluids: CEA, Ca125 and CYFRA 21-1, Se: 29%, 66% and 64% respectively and Sp: 100%, 87% and 100% respectively. b) in sera: CEA, Ca125 and CYFRA 21-1: Se: 27%, 77% and 47% respectively and Sp: 100%, 25% and 75% respectively. *CEA (in cells) + TM (fluids)*: Se 100% and Sp 75% *AgNOR + TM (fluids)*: Se 95% and Sp 87%, *TM Panel (CEA+Ca125+CYFRA 21-1)*: a) in fluids: Se 81% and Sp 87%, b) in sera: Se 86% and Sp 12%. **Conclusion:** AgNOR assay and immunocytochemistry for CEA were useful as complementary tools in the diagnosis using effusions, raising the Sensitivity of the Cytology from 73% to 86% and 96% respectively. Sensitivity increased with the assays for a panel of TM in fluids, but the high cost of these methods does not justify their use for non-conclusive smears.

Key words: Ascitic fluid; Tumor markers, biological; Nucleolus organizer region; Immunohistochemistry

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INTRODUCTION

The production of serous fluids with neoplastic cells in pleura, pericardium or peritoneum has its origin in metastatic tumors mainly, and to a lower extent in primitive mesothelial tumors (mesotheliomas). The evaluation of the smears stained by morphological methods (Papanicolaou and Giemsa) lacks, in many cases, the necessary accuracy to obtain the correct diagnostics[1]. Having in mind the importance of establishing the right result to make the correct decision, it is necessary to develop methods to improve the Sensitivity (Se) (in order to diminish the false-negative cases) and Specificity (Sp) (in order to lower the false-positive cases) in the study of these smears[2].

The low Se of the cytological examination has different reasons: patients who are not shaken before fluid aspiration, resulting in scanty cell smears (especially in ascites); incorrect homogenization of fluids; difficulty in the differentiation between reactive and neoplastic cells especially in long-term effusions. To all these factors we must add the differences between the observers, resulting in a range of Se between 40 to 90 % in the literature, with a certain number of non-conclusive results[3-4].

For this reason, the morphological examination is complemented by other supplementary methods, as immunocytochemistry, Cytogenetics, Molecular Biology, DNA cytometry and ultrastructural studies.

The use of these techniques attempts to add useful criteria to the simple morphological characteristics, especially in the differentiation between adenocarcinomas and reactive mesothelial cells.

The Scanning Electronic Microscope helps in the differentiation among reactive and neoplastic cells (adenocarcinoma or mesothelioma derived cells) because the morphology of the microvillie changes in each case. However, the high cost of this technique does not justify its use in the routine[5-6].

Immunostaining was frequently used to investigate different antigens like Carcinoembryonic Antigen (CEA), Epithelial Membrane Antigen (EMA), Ber-EP4, B72.3, or proteins from oncogenes like the c-erb-2. CEA and EMA are expressed in high percentage in metastatic adenocarcinomatous cells from pleura, pericardium or peritoneum, but some controversies exist about the advantage of using these and other markers in the routine diagnosis[7-8].

The assessment of ploidy is a reliable method for distinguishing between reactive mesothelial cells and malignant (adenocarcinomatous) cells in serous fluids, although this assay needs special staining (Feulgen) and an Image-analyzer[9].

The assay of fibrilar proteins no histones from the nucleolus organizer regions (AgNOR) with silver nitrate, called AgNOR technique, is a good alternative for the differentiation between reactive and neoplastic cells in effusions . This simple and low-cost technique raises the Se in the assessment of malignant effusions[10].

Tumor markers (TM) are being recently used as complementary tools for diagnosis in serous fluids[11]. These TM are investigated in fluids and blood, with the hope that their presence correlates with the existence and growth of tumors. However, some TM have limited value by their low specificity, on account of their expression in benign cells, such as Ca125, expressed in malignant ovarian tumors and in benign pathologies like ovarian cysts, or CYFRA 21-1, used as a TM in lung adenocarcinomas, that is also expressed in some inflammatory pathologies[12]. The circulating TM level in a given patient is the end result of various factors, including the level of gene expression, the rate of TM synthesis, its subsequent release by the tumor, the half-life in the circulation and the degree of vascularization of the tumor. When a TM from the neoplastic cell is not released into the interstitial space and, thence, into the circulation of the tumor-bearing patient, it cannot be detected by immunoassays. The same situation happens when the metastatic cells only infiltrate the submesothelial zone[13]. The reference limit values (cut-off) for each TM must take into account the contribution of all the mentioned factors in the expression of these TM in serum and effusion fluids.

The objective of this study was to evaluate the utility of TM, AgNOR and immunoassays for CEA as complementary tools for diagnosis using serous effusions.

MATERIALS AND METHODS

Fifty-six effusions (26 peritoneal and 30 pleural) and sera from the same patients were studied. The sera and effusion fluids were collected from patients from Pirovano Hospital and the Department of Biochemistry, Cytology Area, Clinical Hospital (University of Buenos Aires), Buenos Aires, Argentina.

The fluids were centrifuged at 1000g for 10 min. From the cells, deposit smears were made and fixed in 96% ethanol. Two of each were stained using the Papanicolaou method. The rest was stored for a) Immunocytochemical assay for Carcinoembryonic antigen (CEA) with maximal fixation time of 60 days at -20 oC; b) Staining with the AgNOR technique.

CEA, CYFRA 21-1 and Ca125 were investigated in the fluid supernatants and in sera from the respective patients.

The cytology diagnosis was recorded as benign, malignant and non-conclusive. The normal mesothelial cells present a cytoplasm with small vacuoles, ruffled border and scanty and small nucleoli. There are "windows" between adjacent cells.

The reactive changes of mesothelial cells are characterized by enlargement of nuclei and nucleoli, clumped chromatin and cellular grouping in sheet, balls or rosettes.

The adenocarcinomatous cells usually show peripheral nuclei with coarser and denser chromatin when compared to mesothelial cells or reactive cells, and also have bigger nucleoli. The groups are three-dimensional, without "windows".

When the cells did not have the mentioned characteristics, the smears were informed as being "non-conclusive".

The final diagnosis was established through evaluation of the clinical history and follow up (during almost 24 months). In some cases pleural or peritoneal biopsies were performed. In 8 cases of pleural effusions and in 7 cases of ascites the first diagnosis of malignancy was made by cytologic examination.

All the malignant effusions were a consequence of metastatic adenocarcinomas; there were no mesotheliomas in our series.

AgNOR Technique: The smears previously fixed in 96% ethanol were incubated in the dark for 25 min with a mix of 5% (W/V) silver nitrate and 1% (W/V) gelatin in 1% V/V (2:1) formic acid. After being washed with deionized water and 1% (W/V) sodium thiosulphate, the slides were dehydrated and mounted using Canada balsam.

The mean value per nucleus was calculated and 100 nuclei counted. The AgNORs were counted only when it was possible to distinguish each individual dot, free or in clusters.

Reference values are: 4.88, s=1.50 for SE with reactive mesothelial cells and 13.78, s=3.89 for SE with neoplastic cells[14].

Immunoassay for CEA: Immunocytochemistry was performed by the labeled streptavidin biotin complex method: after washing with phosphate-buffered saline (PBS), the smears were covered with 3% H2O2 for 5 min. to block endogenous peroxidase activity. After rinsing the slides were incubated for 30 min with the primary antibody: CEA monoclonal (DAKO: Carpinteria, California, USA). The smears were rinsed with PBS and incubated with biotinylated anti-mouse antibody (1:100 DAKO) for 15 min. After another rinse, peroxidase-labelled streptavidin was applied for 15 min (1:100 DAKO). The activity of peroxidase was detected with 0.01% H2O2 and Diaminobenzidine for 15 min. The slides were dehydrated, clarified with xylene and mounted with Canada balsam.

Histological sections from colon adenocarcinoma served as positive controls for CEA.

Omitting the primary antibody made a negative control. The development of a brown-stain into the cytoplasm was taken as positive. In most of the assays the staining was stronger in the cellular membranes.

In many smears a cross-staining with Hematoxylin was carried out[15].

Immunoassay for TM: The effusion fluids and sera of the patients were processed by electrochemiluminescense immunoassay in the Elecsys 2010 autoanalyzer (ROCHE DIAGNOSTIC). The same company provided the controls and the reagents.

The limit values were: (cut-off point); in fluids: CYFRA 100 ng/ml, CEA 40 ng/ml and Ca125 1000 U/ml; in sera: CYFRA 3.3 ng/ml, CEA 3.4 ng/ml and Ca125 35 U/ml.

RESULTS

Thirty-four out of 46 smears of fluids arising from metastasic adenocarcinomas were considered positive for cytology. There were no false positives in this series. The immunocytochemical method for CEA showed high Se, detecting 43/45 of malignant cases, but the marker was expressed in cells of two negative smears (arising from ovarian fibroid and congestive heart failure). The AgNOR was positive ($\geq$ 14 dots / nuclei) in 39/44 malignant smears. For negative fluids, the AgNOR values corresponded to that of reactive mesothelial cells (Table 1).

The TM showed different Se in fluids and in sera, being CEA the less sensitive marker of the panel used. Sp of Ca125 was very low in serum (Tables 2 and 3).

Se of the panel of TMs was high in fluids and sera. Sp of this panel was high in fluids and very low in sera (Fluids: Se 81%, Sp 87% - Serums: Se 86%, Sp 12%). The combination of TM in fluids and immunocytochemistry for CEA showed Se of 100%. Sp remained relatively high (75%). The combination of TM in fluids and AgNOR reached a high Se (95%). The Sp (87%) remained almost unchanged (Table 4).

DISCUSSION

Seventy-three percent of the cases confirmed as malignant fluids were detected by Cytology, and there were no false positive smears in our series (Sp 100%). The smears informed as negative or non-conclusive arise from scanty cell fluids from metastasis of clear cell carcinoma of the kidney, lung carcinoma and unknown primitive tumors. The use of AgNOR increased the Se

Table 1. Cytology, AgNOR and Immunocytochemistry for CEA in Serous Fluids

METHOD	FLUID DIAGNOSIS	NEGATIVE	POSITIVE	IN	ND
CYTOLOGY	Benign	8	--	---	---
	Malignant	---	34	12	2
AgNOR	Benign	8	---	---	---
	Malignant	5	39	---	4
IMMUNOCYT FOR CEA	Benign	6	2	--	---
	Malignant	2	43	---	3

AgNOR Negative: ≤ 5 dots/nuclei
AgNOR Positive: ≥ 14 dots/nuclei
IN: Inconclusive
ND: Not done

Table 2. Expression of Tumor Markers in Serous Fluids

METHOD	FLUID DIAGNOSIS	NEGATIVE	POSITIVE	ND
CEA	Benign	8	--	---
	Malignant	31	13	4
Ca125	Benign	7	1	---
	Malignant	13	32	3
CYFRA 21-1	Benign	8	--	---
	Malignant	14	31	3

CEA Positive: > 40 U/l
Ca125 Positive: >1000 U/l
CYFRA 21-1: > 100 U/l
ND: Not done

Table 3. Expression of Tumor Markers in Serums

METHOD	FLUID DIAGNOSIS	NEGATIVE	POSITIVE	ND
CEA	Benign	8	--	---
	Malignant	32	12	4
Ca125	Benign	2	6	---
	Malignant	10	34	4
CYFRA 21-1	Benign	6	2	---
	Malignant	24	22	2

CEA Positive: >3,4 U/l
Ca125 Positive: > 35 U/l
CYFRA 21-1: > 3,3 U/l
ND: Not done

to 86 %, without changes in Sp (100%). AgNOR is a proliferating marker useful in the differential diagnosis of adenocarcinomatous cells and reactive mesothelial cells. This technique, with the advantage of their low cost and simplicity, decreases the number of non-conclusive cases[14].

The selective use of a small panel of markers for immunocytochemical assays such as MOC-31, Ber-EP4, EMA, CEA, Leu-M1 or BG8 has been proposed to help in the differentiation between reactive cells and adenocarcinomatous cells in serous effusions[16-19].

It was reported that Se of the mentioned markers are lower compared to CEA sensitivity; besides that, taken into account the false-positive reactivity in a high number of cases for some of them (such as Leu-M1) and the controversies about the advantage of the use of these markers, we only investigated CEA expression in our routine immunocytochemical studies[17].

Table 4. Summary of the Results

METHOD	Se	Sp
Smears		
Cytology	73%	100%
Immunocytochemistry for CEA	96%	75%
AgNOR	86%	100%
TM Fluids		
CEA	29%	100%
Ca125	66%	87%
CYFRA	64%	100%
TM Sera		
CEA	27%	100%
Ca125	77%	25%
CYFRA	47%	75%
Combination of Methods		
CEA+Ca125+CYFRA (Fluids)	81%	87%
CEA+Ca125+CYFRA (Sera)	86%	12%
Immunocytochemisty for CEA + TM (Fluids)	100%	75%
AgNOR + TM (Fluids)	95%	87%

Anyway, CEA has some limited value by the low specificity, on account of their expression in benign reactive mesothelial cells. In the present paper, the Sp of CEA was 80%.

Some highly suspect smears of malignant effusions show negative cytology findings. A needle biopsy adds little to cytology[20], and thus an aggressive technique such as thoracoscopy should be considered.

In the last years, the evaluation of TM in serum and serous fluids was carried out in order to improve the effusions diagnosis and to avoid the use of invasive techniques[17-18]. CEA, CYFRA 21-1 and Ca125 are mentioned in a great number of papers[11-12]. CEA is an oncofetal glycoprotein associated to endodermally-derived tumors (lung, gastrointestinal system) and ectodermally-derived tumors (breast). The measurement of CEA levels in pleural effusions yields the highest diagnostic accuracy, in the opinion of some authors[19]. In our series, CEA showed low Se (29% in pleural and peritoneal fluids, 27% in sera), but there were no false positive cases (Sp 100%). Even if we take only pleural fluids, CEA reached pathologic values only in 12/30 cases.

Serum cytokeratin 19 fragments (CYFRA 21-1) have been shown to be the most sensitive TM in non-small cell lung carcinoma, particularly the squamous cell type, although it has some expression in lung adenocarcinomas too. In other malignancies, such as ovarian cancer, the marker was measured in serum samples[12]. In the present paper, pleural levels of CYFRA 21-1 were high in 8/12 (66%) pulmonary neoplasias (data not shown), and taken all the effusions, the Se of this TM was 64 %. The Se of CYFRA 21-1 in serum for all the neoplasias was lower that in fluids (47%).

The Ca125 (Cancer Antigen 125) assay uses a monoclonal antibody that is relatively specific for a surface antigen (mucinous glycoprotein) derived from a papillary serous cystadenocarcinoma. It was used primarily as a marker for cancer of the ovary in serum and serous fluids.

Elevated Ca125 levels can also be seen in association with malignancies of breast, cervix, uterus, liver, pancreas, stomach, colorectum and lung, but non-malignant elevations of Ca125 have been reported for cirrhosis, hepatitis, pancreatitis, fibroids, endometriosis, ovarian cysts, first trimester pregnancy and pelvic inflammatory disease[19].

In this paper, Ca125 reached Se of 77% (similar to that of the Cytology) in serum, but with the lowest Sp for all the tested TM (25%), because of high levels shown in benign pathologies.

The three mentioned TM can be expressed in benign pathologies, resulting in a decrease of Sp. The limit values of the different TM must be raised to avoid the inclusion of the false positive cases, but it results in a loss of Se, as a consequence of losing certain number of tumors with low levels of TM. Moreover, certain tumors are non-secretory for the marker molecules, resulting in false-negative results.

The individual assay for any of the three chosen TM does not contribute too much to the improvement of the effusion diagnosis, but when the panel of TM is considered in fluids, at least one of them showed a pathologic level in 36 neoplastic smears (Se 81%) with relative good Sp (87%). In sera, the results of the panel assay are discouraging in account of the high number of false-positive values (Sp 12%). When the results of the panel of TM in fluids were combined with those of immunocytochemistry for CEA, the Se reached 100% (the two smears negative for immunocytochemistry and the three smears not done showed at least one TM with high value). The Sp remained 75%. Similar improvement is reached combining TM assays in fluids with AgNOR assays. Out of five negatives and four smears not done for AgNOR, seven showed at least one of the TM as high (Se 95%) . The Sp remained 87%, because there were no false-positives in the AgNOR series.

In conclusion, the utility of applying the AgNOR assay and the immunocytochemistry for CEA in cytologic smears in which the diagnosis was non-conclusive was demonstrated. These auxiliary techniques raised the Sensitivity of the Cytology from 73% to 86% and 96% respectively.

Sensitivity increased with the assays for a panel of TM in fluids, but the high cost of these methods does not justify their use as an auxiliary tool for non-conclusive cytologyc diagnosis. The individual assay of any of the investigated TM is a relatively low sensitivity method, that should be limited to confirming the origin of the tumor.

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Resumo

Objetivo: A avaliação de fluidos serosos corados por métodos morfológicos não tem, em muitos casos, a exatidão necessária para se chegar ao diagnóstico correto. O objetivo deste trabalho foi estabelecer o valor de técnicas complementares para a melhoria do diagnóstico, utilizando efusões serosas. **Métodos:** Cinqüenta e seis efusões serosas foram processadas para coloração morfológica, imunocitoquímica do antígeno carcinoembriônico (CEA), contagem de AgNOR e imunoensaios de eletroquimioluminescência para marcadores tumorais (MT): CEA, Ca125 e CYFRA 21-1. Também foram realizados ensaios de MT em soros dos mesmos pacientes. A Sensibilidade (Se) e Especificidade (Sp) foram avaliadas para todos os métodos. **Resultados:** *Citologia:* Se 73%, Sp100%, *CEA por imunocitoquímica:* Se 96%, Sp 75%, *AgNOR:* Se 86%, Sp100%, *MT:* a) em fluidos: CEA, Ca125 e CYFRA 21-1, Se: 29%, 66% e 64%, respectivamente e Sp: 100%, 87% e 100%, respectivamente. b) em soros: CEA, Ca125 e CYFRA 21-1: Se: 27%, 77% e 47%, respectivamente e Sp: 100%, 25% e 75%, respectivamente. *CEA (em células) + MT (fluidos)*: Se 100% e Sp 75%, *AgNOR + MT (fluidos)*: Se 95% e Sp 87%, *Painel de MT (CEA+Ca125+CYFRA 21-1*): a) em fluidos: Se 81% e Sp 87%, b) em soros: Se 86% e Sp 12%. **Conclusão:** O ensaio de AgNOR e a imunocitoquímica para CEA foram úteis como ferramentas complementares no diagnóstico de efusões, aumentando a sensibilidade da citologia de 73% para 86% e 96%, respectivamente. A sensibilidade aumentou com os ensaios para um painel de MT em fluidos, mas o alto custo desses métodos não justifica seu uso para esfregaços inconclusivos.

Palavras-chave: Líquido ascítico; Marcadores biológicos de tumor; Região organizadora do nucléolo; Imunoistoquímica