Accuracy of immunohistochemistry in evaluation of malignant pleural and peritoneal effusions

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Background: The aim was to evaluate the accuracy of immunohistochemistry in diagnosis of the source of malignancies in pleural and peritoneal fluids in comparison to histopathology as the gold standard.
Material and methods: Retrospectively, the cell block specimen and past medical data files of patients who had undergone serosal fluid aspiration and had a histopathology report corresponding to underlying disease were retrieved. Three mesothelial antibodies (D2-40, calretinin and WT-1) and two non-mesothelial antibodies (MOC-31 and EMA) were set to be applied for evaluating malignant cells and benign cells within serous fluids.
Results: Seventy-one patients, 12 men and 59 women, were found to have a thorough information package needed, including cell blocks with appropriate cellularity on which ICC was applicable and the corresponding histopathology report. As mesothelial markers, calretinin and WT-1 were found to have sensitivity and specificity of 90%, 96.7% and 100%, 42.6%, respectively. Sensitivity and specificity of D2-40 both reached 100%. In addition, as non-mesothelial markers, MOC-31 and EMA were demonstrated to have sensitivity and specificity of 95.08%, 90% and 93.4%, 70%, respectively.
Conclusion: D2-40 was reconfirmed to act as an accurate marker in distinguishing between cells of mesothelial and non-mesothelial origin. However, WT-1 is not specific enough to be considered as accurate as D2-40. Considering the sensitivity and specificity of calretinin and MOC-31, they can be considered as safe but not as much as D2-40. EMA is not recommended as an ancillary marker due to its low specificity and challenging results.

Key words: pleural effusion, peritoneal effusion, immunohistochemistry.

Introduction

Malignancies such as lung, breast, gastrointestinal and female genital adenocarcinoma as well as malignant mesothelioma are common sources of effusion in pleural and peritoneal cavities. Histopathology has been used for a long time as the gold standard diagnostic modality. The morphological evaluation, however, is sometimes difficult due to reasons such as a low number of tumour cells, mixed population of tumoural-mesothelial cells and presence of reactive mesothelial cells which mimic the morphology of malignant cells [1-3]. Moreover, mesothelial cells react variously to different sorts of both external and internal stimuli, which can result in misdiagnosis of a malignancy [1, 2]. As finding the source of tumoural cells in serosal fluids is crucial in diagnosis and treatment of patients, substituting histopathology for a more rapid, accurate and cost-effective method seems to be necessary. Therefore, according to difficulties in morphological diagnosis of cancerous serous effusions and considering the critical role of immunohistochemistry, application of a novel panel of diagnostic markers for early and accurate detection of the source

of malignancies in serous fluids is mandatory. As currently used panels are not of acceptable specifications, e.g. sensitivity and specificity, we conducted this study to evaluate the value of a new panel including 3 mesothelial and 2 non-mesothelial markers in detection of tumoural cells within pleural and peritoneal fluids [1, 2].

Material and methods

Patients

Retrospectively and from 2005 to 2008 all patients with pleural and peritoneal effusion for whom histopathological analysis (biopsy from pleura or peritoneum) had been performed, and their pleural/peritoneal fluid cell block were available, were enrolled in the study. Every patient whose result of histopathology was not available, who had inappropriate cell fixation on the cell block and who had insufficient cell count on the cell block was excluded. No age/sex limit was applied.

Laboratory techniques

Available cell blocks were considered for immunohistochemistry. The preparation process of the cell blocks is stated below:

Aspirated pleural/peritoneal fluid was centrifuged for 5 minutes (1000 RPM). The supernatant fluid was discarded whereas the sedimentary material was saved and divided into two halves. Using 96% ethanol, the first half was fixed, from which 4 smears were made and used for cytological study. Cellularity was established by evaluation of haematoxylin and eosin (H&E) stained cell block sections from formalin-fixed paraffin-embedded pellets of the other half, after using 10% buffered formalin as a fixator. If cellularity was confirmed to be sufficient, further characterization by following a 5-antibody panel (using IHC diluent, RE7133 Novocastra, if needed) was carried out:

- D2-40 (1 : 100; Monoclonal mouse antihuman D2-40, Dakocytomation),
- Calretinin (1 : 100; Liquid mouse monoclonal antibody, NCL-L-CaLRET-566, Novocastra),
- WT-1 (1 : 40; Liquid mouse monoclonal antibody, NCL-L- WT-1-562, Novocastra),
- MOC-31 (1 : 50; Mouse monoclonal antibody, Clone = NCL-MOC-31, Novocastra) and
- EMA (1 : 1; Ready to use liquid mouse monoclonal antibody [RTU-EMA], Novocastra).

All cases were stained using Post primary block (Novolink polymer detection system, RE7150-K Novocastra) and Novolink-polymer (Novolink polymer detection system, RE7150-K Novocastra).

Prostate tissue was used as a positive control for D2-40, testis tissue for calretinin, kidney tissue for WT-1, intestinal tissue for MOC-31 and colon or breast tissue for EMA.

D2-40 was interpreted as positive if cytoplasmic and membranous (if any) staining were observed. Calretinin was interpreted as positive if nuclear and cytoplasmic (if any) staining were detected. MOC-31 represented positive results in case membranous staining was seen. WT-1 was interpreted as positive if nuclear staining was observed. And last but not least, EMA was considered positive if cytoplasmic and membranous staining were observed. Each marker was reported as either positive or negative.

Afterwards, each H&E slide as well as all prepared slides regarding positive and negative values for malignant epithelial and mesothelial cells and reactive mesothelial cells was assessed and in the end, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of each marker were generated.

This study was conducted after approval from the institutional board of Tehran University of Medical Sciences.

Results

Serous effusions from 71 patients, 12 (16.9%) men and 59 (83.1%) women (age range: 21-85 y, mean: 53.7 y), from the Department of Cytology, Cancer Institute, Imam Khomeini Medical Complex, Tehran University of Medical Sciences, Tehran, Iran, were retrospectively reviewed. Overall, 14 samples of pleural (19.7%) and 57 peritoneal (80.3%) fluids, including 8 (11.26%) benign and 63 (88.74%) malignant effusions, were collected. From the stated 63 samples of malignant lesions, 14 cases (22.2%) have pleural and 49 (77.8%) have peritoneal origin, 12 (19.4%) cases were from male and 51 (80.6%) cases were from female patients. However, all 8 benign samples were obtained from women. The summary of the involved organs and type of malignancies are given in Tables I and II, respectively.

Table I. Distribution of tumour sources throughout the body

	LUNG	GI TRACT	GENITALIA	BREAST	PLEURA	UNKNOWN ORIGIN	TOTAL
Male	4	4	0	0	2	2	12
Female	5	4	38	2	0	2	51
Total	9	8	38	2	2	4	63

GI tract: gastrointestinal tract

Table II. Histopathological diagnosis of our cancerous population

	ADENO-CARCINOMA NOS	SMALL CELL CARCINOMA	SQUAMOUS CELL CARCINOMA	INVASIVE DUCTAL CARCINOMA	MALIGNANT MESOTHELIOMA	GENITAL SEROUS TUMOUR	GENITAL MUCINOUS TUMOUR	GENITAL ENDOMETRIOID TUMOUR	TOTAL
Male	8	2	0	0	2	0	0	0	12
Female	11	0	2	2	0	34	1	1	51
Total	19	2	2	2	2	34	1	1	63

NOS – not otherwise specified

Immunohistochemical analysis

D2-40

In both (100%) cases of malignant mesothelioma, cytoplasmic and membranous staining was noted (Fig. 1). This included both malignant mesothelial and background reactive mesothelial cells. In all 8 (100%) cases of benign effusions, a positive reaction was noted. However, none of the malignant effusions were positive for this marker. From 63 cases of malignant effusions, 54 cases demonstrated mesothelial reactive cells in the background, among which all (100%) had a positive reaction to D2-40. Sensitivity, specificity, PPV and NPV of this antibody as a mesothelial cell marker all reached 100%.

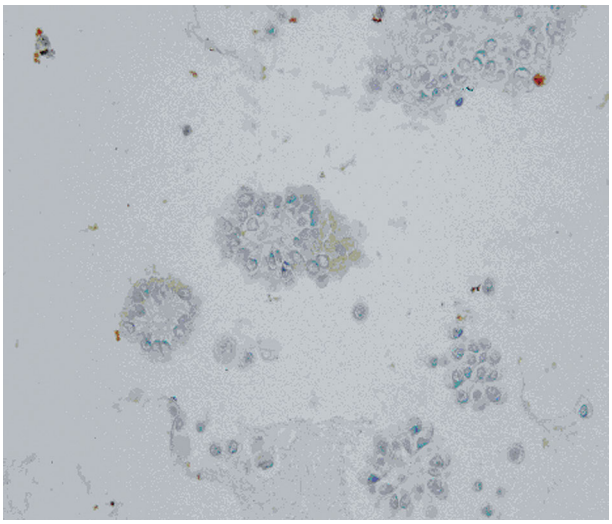

Fig. 1. D2-40 positive malignant mesothelioma. Original magnification 400×

Calretinin

In both (100%) cases of malignant mesothelioma, both malignant and background reactive mesothelial cells showed positive nuclear staining with calretinin. In 2 (3.2%) cases of non-mesothelial tumours (both adenocarcinomas), however, weakly positive cytoplasmic staining was observed (Fig. 2). Out of 8 cases of benign effusions, 7 (87.5%) cases with reactive mesothelial cells were noted to have positive nuclear staining. Moreover, among 63 samples of malignant effusions, 56 cases had background reactive mesothelial cells of which 55 (98.2%) cases presented positive nuclear staining as well. Sensitivity, specificity, PPV and NPV of calretinin as a mesothelial cell marker reached 90%, 96.7%, 81.8% and 98.3%, respectively.

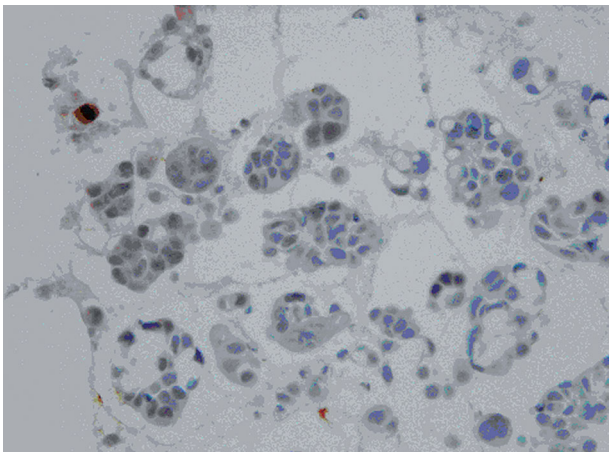

Fig. 2. Calretinin positive papillary serous carcinoma of ovary. Original magnification 400×

WT-1

In both (100%) of our malignant mesothelioma samples, reactive and malignant mesothelial cells showed nuclear staining. Of 61 cases of non-mesothelial tumours, 35 (57.3%) cases showed positive nuclear staining, all being adenocarcinomas, consisting of 32 cases of female genital malignancies, 1 of gastrointestinal and 2 of unknown origin. Additionally, out of 34 cases of female serous genital malignancies, 30 (88.2%) were observed to be positive for WT-1 (Pearson $\chi^2 < 0.0001$). In all 8 (100%) cases of benign effusions, the positive nuclear reaction was remarkable. Of 63 cases of malignant effusions, 53 represented background reactive mesothelial cells, of which 52 (98.11%) had positive staining.

Sensitivity, specificity, PPV and NPV of WT-1 as a mesothelial marker were 100%, 42.2%, 22.2% and 100%, respectively.

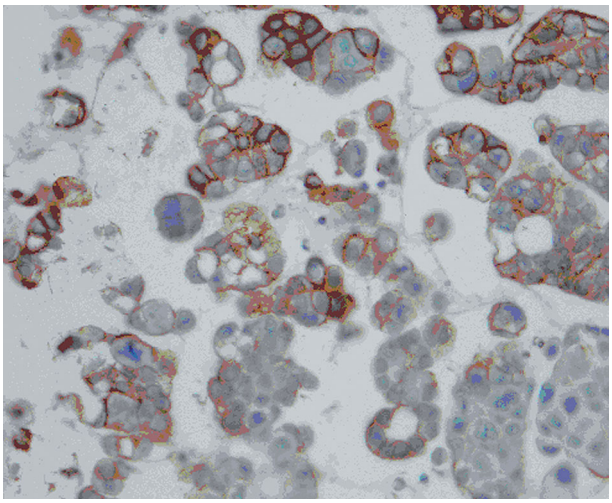

Fig. 3. MOC-31 positive adenocarcinoma. Original magnification 400×

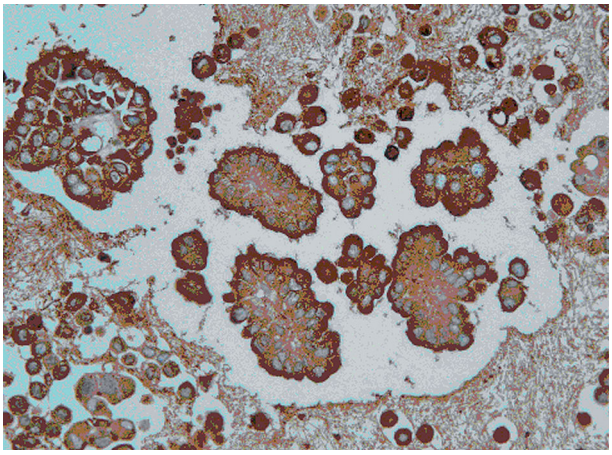

Fig. 4. EMA positive malignant mesothelioma. Original magnification 400×

MOC-31

None of the malignant mesothelioma cases were positive. Among 8 cases of benign effusion, reactive cells of 1 (12.5%) case were noticed to have a clean membranous reaction. 58 (95.08%) out of 61 cases of malignant non-mesothelial samples were shown to have a positive membranous reaction (Fig. 3) which consisted of adenocarcinoma (53 cases), small cell carcinoma (2 cases), squamous cell carcinoma (2 cases) and invasive ductal carcinoma (1 case). Of 63 cases of malignant effusions, 53 cases had background reactive mesothelial cells of which only 2 (3.7%) cases demonstrated positive membranous staining. As a non-mesothelial marker, sensitivity, specificity, PPV and NPV of MOC-31 were 95.08%, 90%, 98.3% and 75%, respectively.

EMA

Both membranous and cytoplasmic positive staining were noted in both (100%) malignant mesothelioma cases regarding both malignant and background reactive mesothelial cells (Fig. 4). Only 1 (12.5%) out of 8 cases of benign effusions had positive cytoplasmic and membranous staining. Out of 61 cases of non-mesothelial malignancies, 57 (93.4%) were noted to have positive membranous and cytoplasmic staining which belong to 52 cases of adenocarcinoma, 2 cases of small cell carcinoma, 2 cases of invasive ductal carcinoma and 1 case of squamous cell carcinoma. Of 63 cases of malignant effusions, 53 cases had background mesothelial cells, of which only 8 (15.09%) cases had positive membranous and cytoplasmic staining. As another non-mesothelial marker, EMA achieved 93.44%, 70%, 95% and 63.6% sensitivity, specificity, PPV and NPV in our study, respectively.

Sensitivity, specificity, PPV and NPV of each ICC marker are summarized in Table III.

Discussion

A variety of benign and malignant disorders can present with serous effusion, of which determination of source and cell behaviour has always been a matter of diagnostic confusion among investigators worldwide. As detection of malignant cells in pleural and peritoneal effusions represents poor prognosis of a patient who, to some extent, suffers from an incurable disease, several immunohistochemical markers have been proposed to help pathologists and clinicians in distinguishing between malignant and benign lesions more accurately.

The aim of this study was to apply a panel of markers which consisted of 3 mesothelial (D2-40, calretinin and WT-1) and two non-mesothelial (MOC-31 and EMA) markers to evaluate if, compared to histopathology, they are accurate enough to distinguish malignant and benign mesothelial and

Table III. Sensitivity, specificity, PPV and NPV of each marker evaluated in this study

	D2-40	Calretinin	WT-1	MOC-31	EMA
Sensitivity	100	90	100	95.08	93.4
Specificity	100	96.7	42.6	90	70
PPV	100	81.8	22.2	98.3	95
NPV	100	98.3	100	75	63.6

PPV – positive predictive value, NPV – negative predictive value

non-mesothelial effusions. This has been performed by other investigators using different panels as well. Having studied 41 cases of metastatic adenocarcinoma and 43 cases of reactive mesothelial cells, Saleh *et al.* demonstrated that MOC-31, BerEp4, HBME-1, and calretinin are an excellent panel of markers for diagnosis, with 97.6% sensitivity for detecting metastatic adenocarcinoma and 90.7% specificity for detecting reactive mesothelial cells [4]. Similarly, Kim and colleagues have evaluated 118 cell blocks of pleural and peritoneal fluids, including 88 cases of adenocarcinoma and 30 benign effusions, regarding diagnosis of reactive mesothelial cells and adenocarcinomas using a panel of MOC-31, D2-40 and calretinin antibody. They concluded that a combination of MOC-31 and D2-40/calretinin is 100% specific and 99% sensitive for diagnosis of adenocarcinomas [5].

We demonstrated that all samples of malignant mesothelioma and benign reactive mesothelial cells had a positive reaction to D2-40. This finding was interestingly similar to some studies performed by other investigators such as Lyons-Boudreaux *et al.* [6] and Bassarova *et al.* [1]. Saleh *et al.,* however, found only 58.1% of their benign effusions to have a positive reaction to D2-40 antibody, though they had no specimens of malignant mesotheliomas [4]. Furthermore, none of our non-mesothelial malignant effusions had a positive reaction to D2-40, which was the same as Lyons-Boudreaux *et al.*'s results [6]. In contrast, using this antibody, Kim *et al.* [5] has reported a single positive case (1.1%) of adenocarcinoma and Bassarova *et al.* [1] has shown positivity in 58% of ovarian carcinomas, 30% of breast carcinomas and 33% of lung carcinomas. Saleh *et al.* [4] and Mimura *et al.* [7] have found 10 cases (24.4%) and 3 cases (4.5%) of their adenocarcinomas with positive membranous staining, respectively. We reached sensitivity, specificity, PPV and NPV of 100% for D2-40 as a mesothelial marker as well as what have been very similarly discovered by Lyons-Boudreaux *et al.* [6] and Kim *et al.* [5]. However, Saleh *et al.* proposed a significantly lower amount of sensitivity and specificity (58.1% and 75.6%, respectively, in detecting reactive mesothelial cell) for this marker [4].

Using calretinin, both (100%) of our malignant mesothelioma cases have shown a positive nuclear reaction, similar to results proposed by Lyons-Boudreaux *et al.* [6] and Politi *et al.* [8]. Furthermore, 2 (3.2%) of our malignant effusions had a positive cytoplasmic reaction to calretinin, which was achieved by Lyons-Boudreaux *et al.* [6], Kim *et al.* [5] and Saleh *et al.* [4] as 2.08%, 2.27% and 29.26%, respectively. Moreover, we found 87.5% of our benign effusions to have a positive reaction to calretinin, which was achieved by Lyons-Boudreaux *et al.* [6], Kim *et al.* [5] and Saleh *et al.* [4] as 58%, 93.3% and 95.3%, respectively. In our study and as a mesothelial marker, sensitivity, specificity, PPV and NPV of calretinin were detected to be 90%, 96.7%, 81.8% and 98.3%, respectively. According to the investigations of Lyons-Boudreaux *et al.* [6], Kim *et al.* [5] and Saleh *et al.* [4], these amounts were 67%, 93% and 95.3% for sensitivity and 98%, 98% and 70.7% for specificity, all respectively. Moreover, Lyons-Boudreaux *et al.* [6] and Kim *et al.* [5] reported PPV and NPV of 94%, 85% and 93%, 98%, respectively, in application of calretinin.

Using WT-1, both of our malignant mesothelioma cases have shown a positive nuclear reaction, while Lyons-Boudreaux *et al.* [6] found 60% positivity. 57.3% of our non-mesothelial tumours have shown nuclear staining, of which 91.4% were of female genital origin. This was reported to be 27% of adenocarcinomas in Lyons-Boudreaux *et al.*'s study [6], which seems to be due to a higher number of cases with serous adenocarcinoma in our study. In our study, all 8 cases with reactive effusion had nuclear staining, which is in contrast to what has been stated in Lyons-Boudreaux *et al.*'s study (50%) [6]. We found sensitivity, specificity, PPV and NPV of 100%, 42.6%, 22.2% and 100% while Lyons-Boudreaux *et al.* proposed these amounts as 52%, 73%, 48% and 76%, respectively [6]. Interestingly, moreover, King and coworkers reached sensitivity and specificity of 77% and 96%, respectively, by performing a systematic review having analysed 88 papers on antibody panels used for distinguishing malignant mesothelioma from metastatic adenocarcinoma [9]. The detected differences between our study and others' regarding calculated sensitivity and specificity in use of WT-1 may be due to a difference in the number of effusions of genital system origin and moreover, in the ratio between benign and malignant effusions.

Using MOC-31, none of our malignant mesothelioma cases have been stained as well as negative results proposed by Lyons-Boudreaux *et al.* [6] and Politi *et al.* [8]. However, Lozano *et al*. had 1 case within their 14 cases of malignant mesothelioma and reactive mesothelial cells with a positive MOC-31 reaction [10]. Regarding benign effusions, we found 1 out of 8 (12.5%) of our patients with positive staining. Lyons-Boudreaux *et al.* [6] and Kim *et al.* [5] had no positive results within their patients using this antibody. 98.05% of our cases with non-mesothelial carcinomatous effusions including small cell carcinoma, squamous cell carcinoma, adenocarcinoma and invasive ductal carcinoma showed positive staining. Meanwhile, other investigators have found similar results for adenocarcinomas as well [5, 6, 8, 10]. In our study, sensitivity, specificity, PPV and NPV of MOC-31, as a non-mesothelial marker, reached 95.08%, 90%, 98.3% and 75%, respectively. However, these amounts for data of Lyons-Boudreaux

et al. [6] and Kim *et al.* [5] were all 100%. Similar to our report, Lozano *et al.* [10] presented sensitivity and specificity of 83.34% and 92.86%, which was a little different from those of Politi *et al*., which were 86.25% and 100%, respectively [8].

Using EMA, cytoplasmic and membranous staining was seen in all cases of malignant mesothelioma, which was similar to that of Shidham's book [11]. Moreover, in our study, out of 8 cases of benign effusions, mesothelioma, 1 received EMA staining only, where as King and coworkers, by performing a systematic review of published papers, stated that positivity for EMA in benign or reactive mesothelium was less than 10% in 2 studies and more than 50% in another [12]. In addition, 93.4% of our non-mesothelial carcinomatous effusions were positive, which consisted of 52 cases of adenocarcinoma, 2 cases of small cell carcinoma, 1 case of squamous cell carcinoma and 2 cases of invasive ductal carcinoma. Sensitivity, specificity, PPV and NPV of our study were 93.44%, 70%, 95% and 63.6% for this marker, respectively. Sensitivity and specificity of EMA used in the project conducted by King *et al.* were 74% and 89%, respectively [12].

According to our findings, none of the applied antibodies were helpful in distinguishing between reactive mesothelial cells and malignant mesothelial cells. On the other hand, D2-40 can be used as a highly sensitive and specific mesothelial marker in serous fluids to distinguish mesothelial cells from others. From this point of view, D2-40 is preferred to calretinin. However, calretinin is a sensitive and specific marker for mesothelial cells.

WT-1 is also a sensitive marker for mesothelial cells but its low specificity limits its application as part of a diagnostic panel. To distinguish adenocarcinomas, MOC-31 is an essentially useful marker of which sensitivity and specificity guarantee its accuracy. In contrast, according to the low specificity of EMA and moreover its challenging results, it would be better not to be considered in diagnostic antibody panels.

We conclude that D2-40 and MOC-31 can be used accurately as two reliable diagnostic components of marker panels. If another study with a maximized sample size is conducted, the results may differ so that more antibodies can be added to the diagnostic panels.

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