New Diagnostic Technique for Rapid Fluorescence Immunocytochemical Staining of Adenocarcinoma and Mesothelial Cells Using Liquid-Based Cytology

(液状処理細胞診を用いた腺癌と中皮の迅速蛍光免疫細胞染色による新しい診断法)

名古屋大学大学院医学系研究科

医療技術学専攻

森 本 亜 由 美

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【緒言】

腹水細胞のLBC検体を用い、酵素抗体法にて腺癌マーカーBer-EP4、CEA、EMA、MOC-31、中皮マーカーcalretinin（monoclonal mouse, monoclonal rabbit）、cytokeratin5/6、desmin、D2-40、HBME-1、mesothelin、thrombomodulin、WT1 の発現を検討した。さらに迅速蛍光抗体法を行い、体腔液細胞診の補助診断としての有用性を検討した。

【対象及び方法】

腹水細胞検体64例（術中腹腔洗浄液35例・腹水29例）を遠心分離して得た沈渣の一部で通常の細胞診断を行い、残りの沈渣をLBC 試薬に保存した。この保存細胞検体（LBC検体）を用いて酵素抗体法を行い、各マーカーの発現を陽性細胞の比率で、－：0%，±：＜10%，＋：10ー50%，＋＋：＞50% の －から＋＋の4段階に評価し、比較した。迅速蛍光抗体法は、腺癌マーカーBer-EP4 を用いて保存細胞液中で抗体反応を行い、通常の蛍光抗体法と比較した。

【結果】

腺癌マーカーBer-EP4、CEA、EMA、MOC-31 の陽性率は腺癌例92%（12／13）、腺癌疑い例57%（4／7）、陰性例5%（2／44）であった。一方中皮マーカーcalretinin monoclonal mouse、calretinin monoclonal rabbit 、cytokeratin5/6 、desmin 、D2-40 、HBME-1 、mesothelin 、thrombomodulin、WT1 の陽性率は、腺癌例8ー15%（1ー2／13）、腺癌疑い例43ー57%（3ー4／7）、陰性例93ー95%（41ー42／44）であった。Ber-EP4 を用いた蛍光抗体法は、通常法、迅速法いずれも酵素抗体法で診断された腺癌細胞陽性18例全例で同様の陽性細胞を認めた。一方、腺癌細胞陰性46例には陽性細胞を認めなかった。

【考察】

LBC を用いた酵素抗体法、蛍光抗体法はともに有用な結果を得ることができた。特に迅速蛍光抗体法は、現在主にパパニコロウ染色、ギムザ染色のみで行われている術中迅速腹水細胞診検査に併用して用いることで、より高い診断精度が得られることが示唆される。

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Abstract

Objective

To evaluate the expression of antibodies against calretinin, cytokeratin5/6, desmin, D2-40, HBME-1, mesothelin, thrombomodulin, WT1, Ber-EP4, CEA, EMA and MOC-31 individually and to compare it with the rapid procedure for fluorescence immunocytochemical staining using liquid-based cytology (LBC).

Study Design

Sixty four peritoneal cell specimens prepared with LBC method were stained with these markers to evaluate its usefulness, and we have developed the rapid procedure for fluorescence immunostaining method using Ber-EP4 that is applicable to intraoperative cancer cytodiagnosis.

Results

The adenocarcinoma markers showed positive in 92% of adenocarcinoma cases, 57% of suspicion of adenocarcinoma cases, and 5% of negative cases (reactive mesothelial cells). On the other hand, the mesothelial cell markers showed positive in 8 to 15% of adenocarcinoma cases, 43 to 57% of suspicion of adenocarcinoma cases, and 93 to 95% of negative cases. With the rapid procedure for fluorescence immunocytochemical staining, we could clearly stain only the adenocarcinoma cells for a limited time.

Conclusion

Immunocytochemical examination with LBC method is a powerful ancillary technique for distinction of adenocarcinoma cells from mesothelial cells. Rapid procedure for fluorescence immunocytochemical staining can be used as an ancillary technique for detecting adenocarcinoma cells in the intraoperative cytological examination of peritoneal or pleural washing fluid.

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Ayumi Morimoto, C.T., I.A.C.[a] Akihiro Ito, M.D.[b] Katsunori Hashimoto, C.T., Ph.D., I.A.C.[c] Akinobu Nakano, M.D., Ph.D.[a] Tetsuro Nagasaka, M.D., Ph.D.[c] and Toyoharu Yokoi, M.D., Ph.D., F.I.A.C.[d]

[a] Department of Clinical Laboratory, Toki General Hospital, Toki, Gifu, Japan, [b] Department of Surgery, Toki General Hospital, Toki, Gifu, Japan, [c] Department of Pathophysiological Laboratory Sciences, Nagoya University Graduate School of Medicine, Nagoya, Japan, and [d] Department of Diagnostic Pathology, Aichi Medical University Hospital, Nagakute, Aichi, Japan

Correspondence to: Dr. Ayumi Morimoto

Department of Clinical Laboratory, Toki General Hospital, 703-24 Tokiguchi, Tokitsucho, Toki, Gifu 509-5193, Japan

Tel. +81 572 55 2111, Fax +81 572 53 0074, E-Mail a.morimoto@mbr.nifty.com

Key Words

Adenocarcinoma · Mesothelial cell · Fluorescence immunocytochemistry · Liquid-based cytology

Abstract

Objective: To evaluate the expression of antibodies against calretinin, cytokeratin5/6, desmin, D2-40, HBME-1, mesothelin, thrombomodulin, WT1, Ber-EP4, CEA, EMA and MOC-31 individually and to compare it with a new rapid procedure for fluorescence immunocytochemistry (ICC) using liquid-based cytology (LBC). ***Study Design:*** Sixty-four peritoneal cell specimens prepared with the LBC method were stained with these markers to evaluate their usefulness, and develop a rapid fluorescence immunostaining method using Ber-EP4 that is applicable to intraoperative cancer cytodiagnosis. ***Results:*** The adenocarcinoma markers showed positive in 92% of adenocarcinoma cases, 57% with suspicion of adenocarcinoma cases, and 5% of negative cases (reactive mesothelial cells). On the other hand, the mesothelial cell markers showed positive in 8 to 15% of adenocarcinoma cases, 43 to 57% with suspicion of adenocarcinoma cases, and 93 to 95% of negative cases. The rapid new fluorescence ICC procedure clearly stained only the adenocarcinoma cells within 20 minutes. ***Conclusion:*** Immunocytochemical examination with the LBC method is a powerful ancillary technique for discriminating adenocarcinoma cells from mesothelial cells. This rapid new fluorescence ICC procedure can be used as an ancillary technique for accurate detection of adenocarcinoma cells in the intraoperative cytological examination of peritoneal or pleural washing fluid.

Introduction

Cytological diagnosis of malignant effusions is often difficult mainly because of the presence of reactive mesothelial cells. Differentiation of malignant cells, especially adenocarcinoma cells, from reactive mesothelial cells has been the most common and serious diagnostic difficulty in effusion cytology [1, 2, 17]. In addition to the conventional body fluid cytology, intraoperative cytological examination of peritoneal or pleural washing fluids has been recently introduced for rapid assessment of the clinical stage of the patient. Intraoperative cytological examination of peritoneal washing fluid is now listed as one of the prognostic factors and recommended to perform according to the Japanese Classification of Gastric Carcinoma [3]. Adenocarcinoma cells in the peritoneal washing fluid are considered an important factor for peritoneal dissemination when making a prognosis. This information is considered crucial for surgeons so as to perform operations according to the most reliable detection techniques.

Immunocytochemistry (ICC) has been applied to aid the differentiation of adenocarcinoma and reactive mesothelial cells [1, 2, 4], but is not very useful largely because of the limited number of specimens, in contrast to immunohistochemistry, in which a sufficient number of consecutive sections are available for each tissue specimen. Liquid-based cytology (LBC) systems have been widely applied not only to gynecological cytology but also to non-gynecological cytology [5, 6, 7]. LBC is superior to conventional cytology in that it provides smear slides with a uniform cell density and a distribution that enables a precise cytological examination. Moreover, one can preserve cells in the LBC solution for a long period of time and always utilize it for a variety of purposes, such as ICC and molecular analysis.

Over the years, many different markers have been tried to elucidate difficult cases. Based on these experiences and more recent literature findings, a test panel of 13 antibodies was selected, which was supposed to be capable of differentiating between adenocarcinoma cells and reactive mesothelial cells. The immunocytochemical markers, Ber-EP4, CEA, EMA and MOC-31 have been reported to be highly expressed in adenocarcinomas or epithelial tumors in general and not in reactive mesothelial cells. The other markers, calretinin (mouse monoclonal and rabbit monoclonal antibody), cytokeratin5/6, desmin, D2-40, HBME-1, mesothelin, thrombomodulin and WT1, are thought to be those of mesothelial cells, although their specificities vary depending on the marker.

In the present study, we performed immunocytochemical analysis on 64 peritoneal cell specimens prepared with the LBC method to evaluate its usefulness, and we developed a new and rapid procedure for fluorescence immunostaining method that is applicable to intraoperative cancer cytodiagnosis.

Materials and Methods

Clinical Materials

Sixty-four peritoneal cell specimens, 35 intraoperative peritoneal washing fluids and 29 peritoneal effusions, were studied at the Department of Pathology, Toki General Hospital, from 2008 to 2010 (table 1). Patients were 38 men and 26 women aged 35 to 88 years (mean 68.6). Peritoneal cell specimens were centrifuged and an aliquot of cell pellet was prepared for conventional Papanicolaou, PAS and Giemsa-stains, then fixed in 95% ethanol more than 15 minutes and processed for routine cytomorphologic

evaluation (table 1). After sampling the routine materials, the rest of the cell pellet was suspended in the LBC solution (Liqui-PREP™ Preservative Solution, LGM International Inc.) for fixation and preservation.

Preparation of LBC Slides for Papanicolaou-stain and ICC

An aliquot of cells preserved in LBC solution was centrifuged and the cell pellet was suspended in Cellular Base Solution (Liqui-PREP™, LGM International Inc.), dropped on the glass slide and dried at room temperature when used for Papanicolaou-stain and ICC. After microwave heat treatment for 15 minutes (5min/3times) in 10 mmol/L citrate buffer (pH 6.0), the cell specimens were cooled at room temperature. Next, the cell specimens were treated with 0.3% hydrogen peroxide in methanol for 20 minutes at room temperature to block endogenous peroxidase activity, and the nonspecific reaction was blocked by treating with 10% normal goat serum (Nichirei, Tokyo, Japan) for 20 minutes at room temperature. Then, cell specimens were incubated with the primary antibodies listed in Table 2 for overnight at 4°C. Subsequently, the specimens were reacted with Histofine reagent (Nichirei, Tokyo, Japan) for 30 minutes at room temperature, and were visualized by DAB chromogen (Vector Laboratories, Inc.) for 15-360 seconds at room temperature. Finally, the cell specimens were immersed in distilled water for stopping the reaction, and were counterstained with Mayer's hematoxylin (fig. 1). Immunocytochemical expressions of the markers were evaluated according to the proportion of positive cells as follows: no staining, ±: less than 10%, +: 10-50%, ++: >50% independent of the results of the conventional cytomorphologic evaluation.

Rapid Procedure for Fluorescence ICC

This new procedure was performed on cells preserved in LBC solution using the liquid-phase method to save time. An aliquot of preserved cell solution was incubated with the primary antibody Ber-EP4 (1%) and centrifuged for 5 minutes at room temperature concurrently. In this step, the antigen-antibody reaction is thought to fully develop during centrifugation. Then, the cell pellet was mixed with the 1% FLUORESCEIN ANTI-MOUSE IgG (Vector Laboratories, Inc.) in PBS, and centrifuged for 5 minutes at room temperature during which a secondary antigen-antibody reaction develops likewise. Subsequently, the cell pellet was suspended in Cellular Base Solution, dropped on the glass slide and dried with a dryer, and mounted with fluorescence mounting medium containing a blue counterstain (VECTASHIELD® Mounting Medium with DAPI, Vector Laboratories, Inc.). The specimen was observed with a fluorescence microscope (OLYMPUS PROVIS AX80+DP-70) (fig. 2). The cytoplasm of positive cells was stained green by FITC (fig. 3).

Standard Fluorescence ICC

To compare the sensitivity and specificity, we performed the standard fluorescence ICC on cytology slides prepared from cells preserved in LBC solution. After 10% normal goat serum treatment for 20 minutes at room temperature, the cell specimens were incubated with the primary antibody Ber-EP4 for 1 h at room temperature. Then, the cell specimens were incubated with the 1% FLUORESCEIN ANTI-MOUSE IgG in PBS for 1 h at room temperature. Subsequently, the cell specimens were mounted with fluorescence mounting medium containing a blue counterstain, and observed with a fluorescence

microscope the same as used for the rapid procedure for fluorescence ICC.

Results

Cytological Features of LBC Slides

There was no staining quality problem compared to the conventional Papanicolaou-stain slides. Cytomorphologic evaluation of the LBC specimens showed adenocarcinoma in 13, suspicion of adenocarcinoma in 7, and negative (reactive mesothelial cell) in 44, the same as the results of the conventional method (table 1).

Standard ICC

Results of the immunocytochemical examination were shown in Table 3. The adenocarcinoma marker Ber-EP4, CEA, EMA and MOC-31 showed positive in 12 (92%) of 13 adenocarcinoma cases, 4 (57%) with 7 suspicion of adenocarcinoma cases, and 2 (5%) of 44 negative cases (reactive mesothelial cells). On the other hand, the mesothelial cell marker calretinin mouse monoclonal antibody, calretinin rabbit monoclonal antibody, cytokeratin5/6, desmin, D2-40, HBME-1, mesothelin thrombomodulin and WT1 showed positive in 1 to 2 (8-15%) of 13 adenocarcinoma cases, 3 to 4 (43-57%) of 7 suspicious adenocarcinoma cases, and 41 to 42 (93-95%) of 44 negative cases (reactive mesothelial cells). With the ICC using LBC specimens, it was apparent that 18 cases were positive for adenocarcinoma cells and 46 cases were negative from all 64 cases. Moreover, it proved that a small amount of adenocarcinoma cells

was intermingled in 2 of the 44 negative cases. In conclusion, in these 64 cases, solely cytomorphologic evaluation with Papanicolaou-stain of sensitivity for the detection of adenocarcinoma was 89% (16 of 18), specificity was 91% (42 of 46) and diagnostic accuracy was 91% (58 of 64).

Rapid Procedure for Fluorescence ICC

To quickly distinguish adenocarcinoma cells from mesothelial cells, the rapid fluorescence ICC procedure was performed on LBC samples. On the basis of the immunocytochemical results described above, Ber-EP4 was selected as an adenocarcinoma marker because it stained the most clearly without any nonspecific reaction. As shown in Figure 4, adenocarcinoma cells were readily separated by fluorescence coloration with the antibody for Ber-EP4. Results of the rapid procedure for fluorescence ICC were the same as those of the standard fluorescence ICC (table 4). Using the rapid procedure for fluorescence ICC, we could clearly stain only the adenocarcinoma cells within 20 minutes in 18 of the adenocarcinoma cases.

Discussion

A wide variety of immunocytochemical markers have been applied to distinguish adenocarcinoma cells from reactive mesothelial cells or malignant mesothelioma in body fluid effusion cytology or serosal histology [8, 9]. CEA is probably the most popular marker for adenocarcinoma, and has been widely used because of its high specificity [8]. In this study, adenocarcinoma cells showed strong expression of CEA, as shown in previous reports, reconfirming that this is an excellent marker for adenocarcinoma. However, the

presence of a nonspecific reaction to leukocytes often makes precise evaluation difficult. This is why other markers have been sought as more specific markers for adenocarcinoma. The expression of EMA is seen in epithelial tumors in general and also in some nonepithelial tumors. This marker is known to be expressed in malignant mesothelioma cells, by its strong, predominantly membranous staining pattern; however, when its presence in a predominantly cytoplasmic staining pattern is indicative of an adenocarcinoma, it is used in the crucial differential diagnosis of malignant mesothelioma cells and adenocarcinoma cells [10]. Despite an expression in a fairly wide spectrum of tumors and nontumorous cells, EMA is still useful for distinguishing adenocarcinoma cells from mesothelial cells in effusions, as shown in this study and in previous reports. MOC-31 and Ber-EP4 are rather new markers introduced into the field of diagnostic pathology and cytology practice [1, 8, 11, 12]. MOC-31 is reported to be fairly specific for adenocarcinoma, while Ber-EP4 is more widely expressed in carcinomas with glandular or some other differentiations. The usefulness of these markers in the differential diagnosis of adenocarcinoma cells and mesothelial cells was confirmed in this study, showing strong expressions of these markers in adenocarcinoma cells and complete absence of expressions in mesothelial cells. Given their high specificity and sensitivity, Ber-EP4 was adopted as a marker for adenocarcinoma in the rapid fluorescence ICC procedure in our study. Contrary to those for adenocarcinoma, there have been few positive markers for mesothelial cells until recently. HBME-1, thrombomodulin and mesothelin, which have been used for a relatively long time, show a high sensitivity to mesothelial cells, but in a sizable percentage of adenocarcinoma cases they are also seen in the expression of these markers [13, 14]. Therefore, use of HBME-1, thrombomodulin and mesothelin as specific markers for mesothelial cells has been limited to special occasions. Calretinin, cytokeratin5/6,

D2-40 and WT1 are recently developed markers reported to be more specific than those mentioned above [9, 15, 16]. Desmin is originally a marker for muscle cells and also reported to be expressed in reactive mesothelial cells but not in mesothelioma cells, thus being used for differentiation of the latter two cells [17]. In the present study, mesothelial cells showed relatively strong positivities for these markers. By preserving peritoneal cell specimens in LBC solution, ICC can obtain almost all of the possible antibodies necessary for diagnosis. We therefore achieve a high degree of diagnostic accuracy compared to a cytomorphologic evaluation with only conventional Papanicolaou, PAS, or Giemsa-stain. However, it is costly to perform many kinds of ICC on all cases. To be more cost-effective, one must carefully observe the conventional Papanicolaou, PAS, or Giemsa-stain.

To improve diagnostic accuracy, ICC or fluorescence in situ hybridization (FISH) of intraoperative cancer diagnosis has been performed in various organs. However, its application to effusion cytology is not common, especially in fluorescence ICC [18-21]. In this study, we conducted the rapid procedure for fluorescence ICC using Ber-EP4 that can be performed within 20 minutes using the liquid-phase method. Although the mechanism by which the antigen-antibody reaction time is shortened by the liquid-phase method has not yet been fully clarified, we found out that it yielded the same results of both standard ICC and standard fluorescence ICC. The combined use of our rapid procedure for fluorescence ICC technique with other staining proved useful for improving intraoperative cytological diagnosis. This procedure can be performed much more easily with LBC specimens. When a conventional Papanicolaou stained-slide is examined and immunostaining is indicated, decoloration of the Papanicolaou-slide is usually unavoidable unless additional slides are incidentally preserved in ethanol. The LBC system is superior in that one can

preserve cells for one or two months in an optimal state without losing antigenicity in the cells and even for more than one year in a fairly adequate state. Thus, cells preserved in LBC solution can be used not only for ICC but also for FISH or other molecular techniques at the same time as or after Papanicolaou, Giemsa or some other conventional stains.

In conclusion, immunocytochemical examination with LBC method is a powerful ancillary technique for differentiation of adenocarcinoma cells from mesothelial cells in body fluid effusion cytology. The rapid new procedure for fluorescence ICC can be used as an ancillary technique for detecting adenocarcinoma cells in the intraoperative cytological examination of peritoneal or pleural washing fluid with a high degree of diagnostic accuracy. The LBC method is an excellent cell preparation technique that can be applied to various diagnostic and research techniques.

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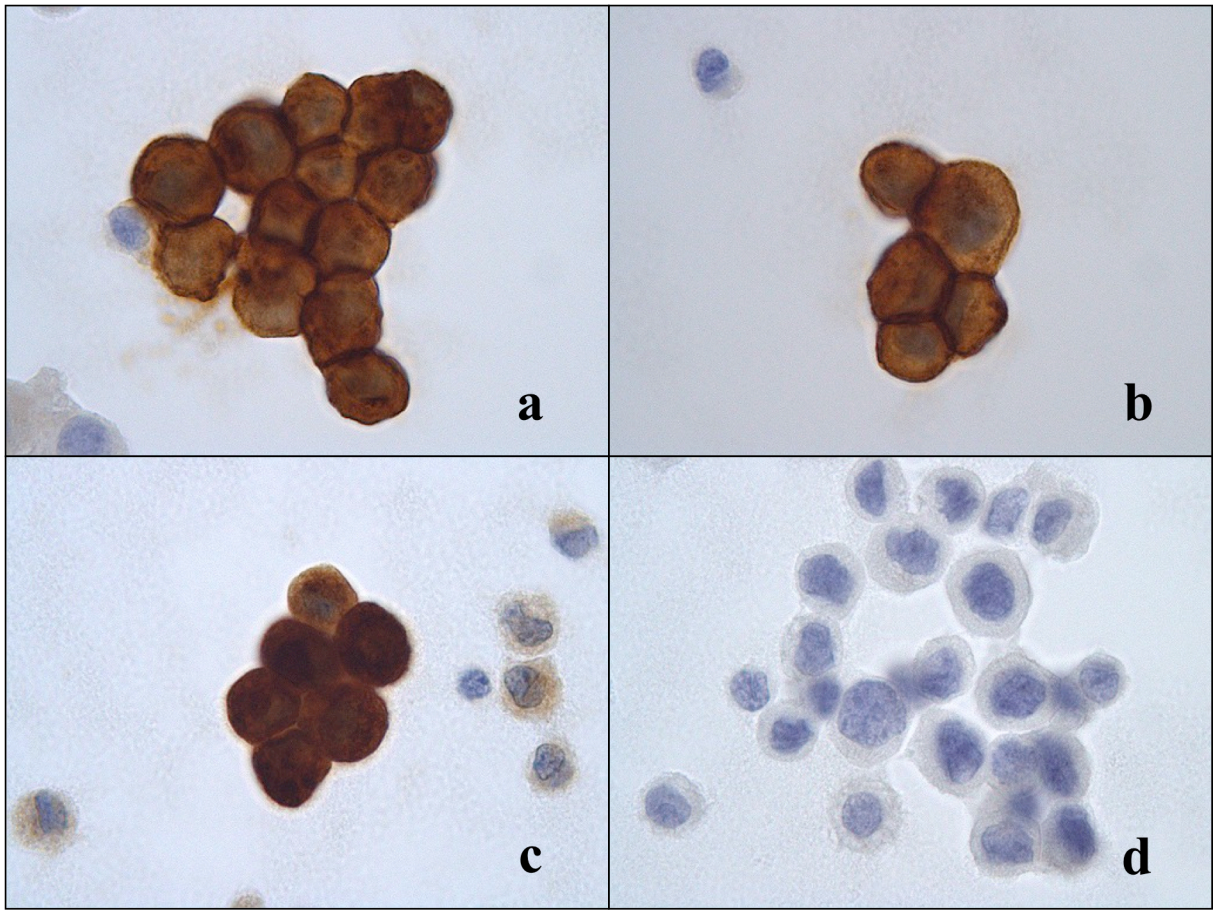

Fig. 1. Typical microscopic appearance of immunocytochemical staining of LBC slides from patient 14. Adenocarcinoma cells were positive for Ber-EP4 (a), MOC-31 (b), and EMA (c), but negative for calretinin mouse monoclonal antibody (d) ×1000.

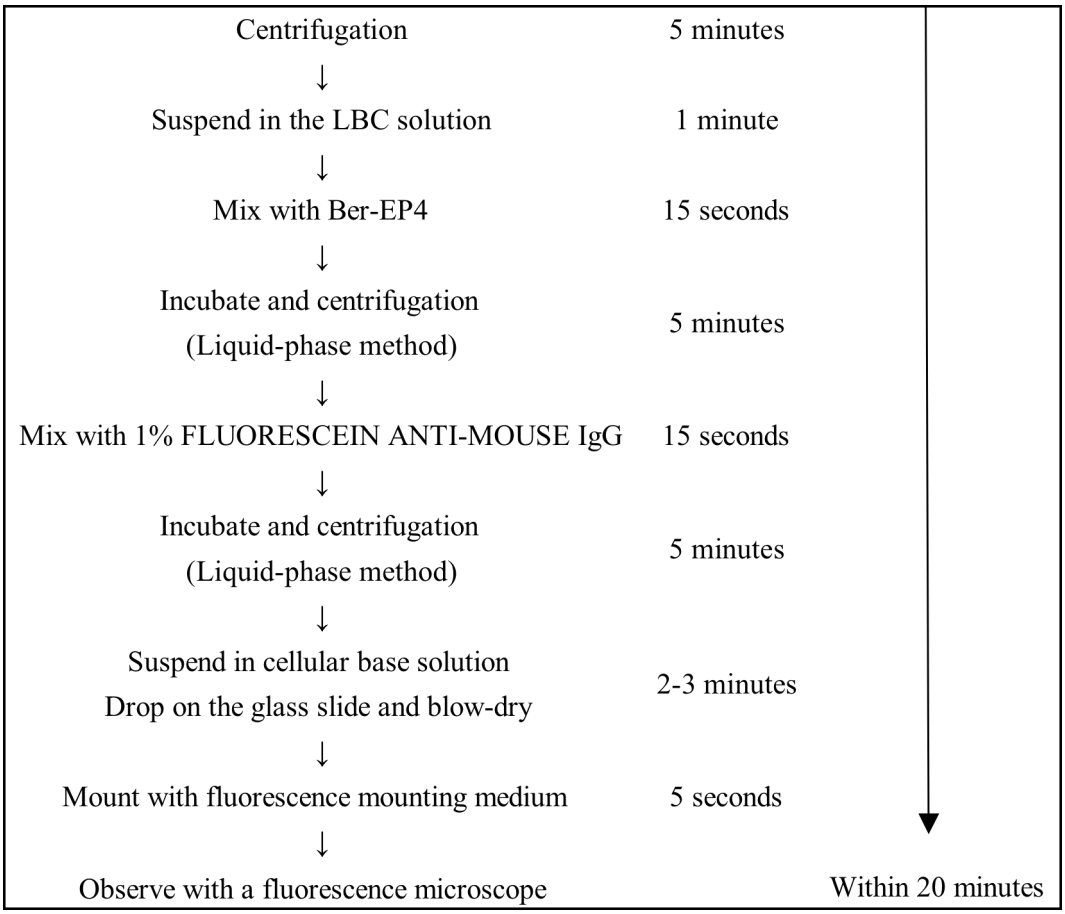

Fig. 2. Rapid procedure for fluorescence immunocytochemical staining.

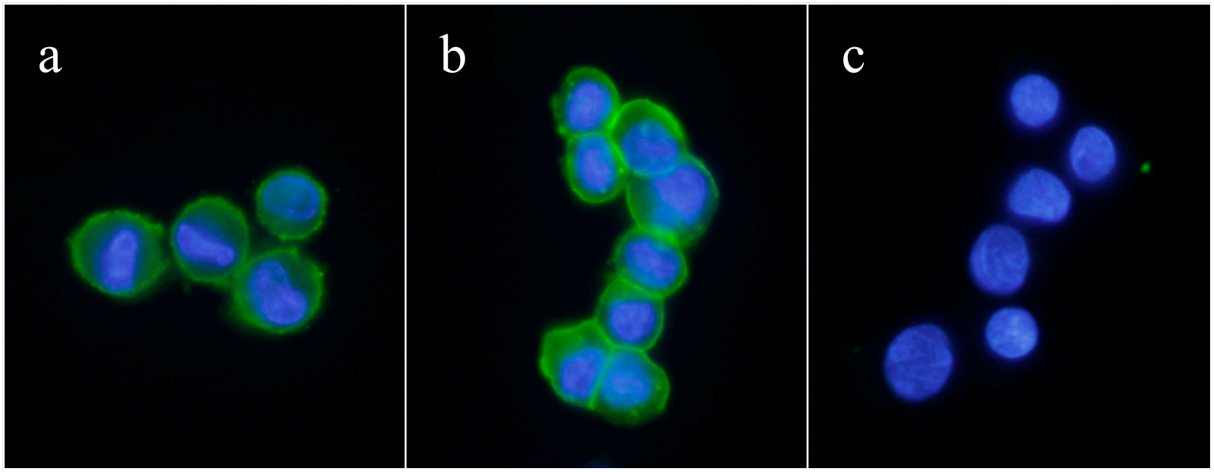

Fig. 3. Rapid procedure for fluorescence immunocytochemical staining. Adenocarcinoma cells were positive for Ber-EP4 (a), (b), and mesothelial cells were negative (c). (a), patient 2; (b), patient 14; (c), patient 28 ×1000.

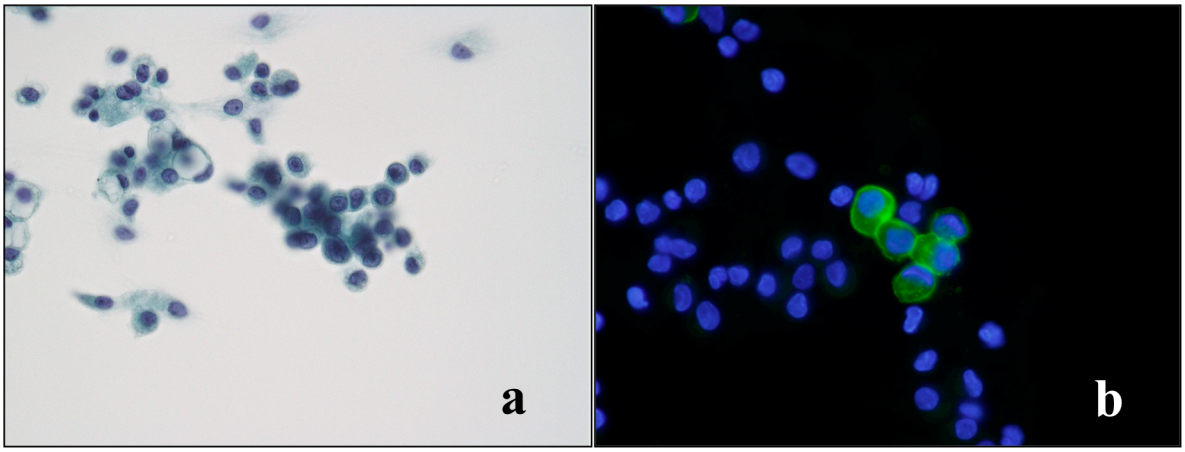

Fig. 4. Typical microscopic appearance of conventional Papanicolaou-stained smear slide (a) and rapid procedure for fluorescence immunocytochemical staining of LBC slides (b) from patient 14. Adenocarcinoma cells were positive for Ber-EP4 (b) ×400.

Table 1. Clinical and cytomorphologic evaluation of 64 peritoneal cell specimens

Patient	Age	Sex	Specimen	Preliminary diagnosis with Pap. Stain	Final diagnosis	Primary disease
1	45	F	W	Adenocarcinoma	Adenocarcinoma	Gastric cancer
2	57	F	E			
3	74	M	W			
4	73	F	E			
5	74	F	E			
6	46	F	W			
7	67	F	E			Pancreatic cancer
8	54	M	E			
9	65	M	W			Colorectal cancer
10	77	M	W			
11	74	M	W			
12	88	F	E			Unknown
13	80	M	W		Mesothelial cell	Gastric cancer
14	70	F	E	Suspicious adenocarcinoma	Adenocarcinoma	Gastric cancer
15	75	F	E			
16	72	F	W			
17	69	M	W			Colorectal cancer
18	75	F	E		Mesothelial cell	Gastric cancer
19	71	F	W			
20	73	M	W			Colorectal cancer
21	70	F	E	Mesothelial cell	Adenocarcinoma	Gastric cancer
22	82	M	E			Cholangiocarcinoma
23	56	M	W		Mesothelial cell	Gastric cancer
24	75	M	W			
25	35	M	W			
26	72	M	W			
27	76	F	W			
28	75	M	E			Pancreatic cancer
29	75	F	E			Cholangiocarcinoma
30	58	F	W			Endometrial cancer
31-51	52-87	M(17), F(4)	W(17), E(4)			Colorectal cancer
52	70	F	E			Liver cirrhosis
53	45	M	E			
54	56	M	E			
55	53	M	E			
56	52	F	W			Ileus
57	69	M	E			Malignant lymphoma
58	60	M	E			
59	59	F	E			Renal failure
60	57	F	E			Nephrotic syndrome
61	80	M	E			COPD
62	72	F	E			Unknown
63	40	F	E			
64	75	M	E			

W, intraoperative peritoneal washing fluid; E, peritoneal effusion.

Table 2. List of antibodies used

Antibody	Clonality	Clone	Dilution	Antigen retrieval	Source
Ber-EP4	M (Mouse)	Ber-EP4	1 : 100	Microwave, citrate buffer, pH 6.0	Dako
CEA	P (Rabbit)	CEA	1 : 800	None	Dako
EMA	M (Mouse)	E29	1 : 100	None	Dako
MOC-31	M (Mouse)	MOC-31	1 : 60	Microwave, citrate buffer, pH 6.0	Dako
Calretinin	M (Mouse)	DAK Calret 1	1 : 50	Microwave, citrate buffer, pH 6.0	Dako
Calretinin	M (Rabbit)	SP13	1 : 50	Microwave, citrate buffer, pH 6.0	Nichirei
Cytokeratin 5/6	M (Mouse)	D5/16 B4	1 : 50	Microwave, citrate buffer, pH 6.0	Dako
Desmin	M (Mouse)	D33	1 : 100	Microwave, citrate buffer, pH 6.0	Dako
D2-40	M (Mouse)	D2-40	1 : 50	Microwave, citrate buffer, pH 6.0	Dako
HBME-1	M (Mouse)	HBME-1	1 : 50	None	Dako
Mesothelin	M (Mouse)	5B2	1 : 20	Microwave, citrate buffer, pH 6.0	Novocastra
Thrombomodulin	M (Mouse)	1009	1 : 50	Microwave, citrate buffer, pH 6.0	Dako
WT1	M (Mouse)	6F-H2	1 : 100	Microwave, citrate buffer, pH 6.0	Dako

M, monoclonal; P, polyclonal.

Table 3. Results of immunocytochemical expression of various markers

Preliminary diagnosis with Pap. stain	Expression	Ber-EP4 / MOC-31	CEA / EMA	Calret (M)	Calret (R)	CK5/6	Desmin HBME-1	D2-40	Meso	TM	WT1
AC (13)	–	1	1	12	12	11	12	12	11	11	12
	±	0	0	0	0	0	0	0	1	1	0
	+	0	1	1	0	1	0	0	0	0	0
	++	12	11	0	1	1	1	1	1	1	1
Suspicious AC (7)	–	3	3	4	3	4	4	4	4	3	4
	±	0	0	0	0	0	0	0	0	1	0
	+	0	0	1	2	0	0	1	1	1	0
	++	4	4	2	2	3	3	2	2	2	3
Mesothelial cell (44)	–	42	42	2	2	2	2	2	2	2	2
	±	0	0	0	0	0	0	0	1	0	0
	+	0	0	10	4	7	0	2	8	3	1
	++	2	2	32	38	35	42	40	33	39	41

AC, adenocarcinoma; Calret (M), calretinin mouse monoclonal antibody; Calret (R), calretinin rabbit monoclonal antibody; CK5/6, cytokeratin5/6; Meso, mesothelin; TM, thrombomodulin. –: no staining, ±: less than 10%, +: 10-50%, ++: >50%.

Table 4. Comparison of three immunocytochemical methods

Preliminary diagnosis with Pap. stain	Expression	ICC	FICC	RFICC
AC (13)	–	1	1	1
	±	0	0	0
	+	0	0	0
	++	12	12	12
Suspicious AC (7)	–	3	3	3
	±	0	0	0
	+	0	0	0
	++	4	4	4
Mesothelial cell (44)	–	42	42	42
	±	0	0	0
	+	0	0	0
	++	2	2	2

AC, adenocarcinoma; ICC, standard immunocytochemistry; FICC, standard fluorescence immunocytochemistry; RFICC, rapid procedure for fluorescence immunocytochemistry. –: no staining, ±: less than 10%, +: 10-50%, ++: >50%.