J Med Dent Sci 2020; 67: 21−29

Original Article

Application of cell block preparation in effusion cytology: Analysis of mismatched diagnosis and utility of immunostaining

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Abstract

Objective: To analyze the mismatched cases between liquid-based cytology (LBC) and cell block in effusion cytology and to confirm the utility of cell block.

Methods: One hundred eighty-two samples of effusions were examined. Cell blocks were prepared from residual samples after LBC preparation, and the details about the diagnostic concordance and difference in cytological characteristics were investigated. Cell block immunostaining was performed to predict the histological type and the primary site of the carcinoma in 32 cases. *ALK* rearrangement and *EGFR* mutation were also analyzed using the cell block.

Results: The diagnostic concordance rate between LBC and cell block was 97.3%. Diagnoses using LBC and cell block were mismatched in five cases. By immunostaining, the histological type was determined in 91.0% of carcinomas, and primary sites were identified in 76.5% of adenocarcinomas. *ALK* rearrangement was examined in two cases of lung carcinoma and *EGFR* mutation was examined in four cases of lung carcinoma.

Conclusion: A high concordance rate between LBC and cell block in effusion cytology was found. The main cause of the mismatched diagnosis was the small amount of atypical cells in LBC or cell block. We also showed the utility of cell block in immunostaining and DNA analysis.

Key word: cell block, liquid-based cytology, pleural effusion, peritoneal effusion, immunohistochemistry.

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Received October 3 ; Accepted December 27, 2019

Introduction

Effusion cytology is an important examination used not only to detect atypical or malignant cells, but also to determine the staging, clinical treatment, and prognosis of cancer[1, 2]. For example, the result of peritoneal effusion cytology for gastric cancer and ovarian tumor directly leads to the determination of cancer staging. The detection rate of malignancy is generally lower in histology than in cytology, because the histological examination is often difficult to perform unless the lesions are not anatomically identified. In this case, the final diagnosis is often made by cytological examination alone. In effusion cytology, we should try to determine the histological type and predict the primary site. Additional examinations such as immunocytochemistry and genetic analysis are usually difficult to perform with cytological samples, because of the short-term preservation and limitation of sample quantity. In such cases, additional preparation of cell block is helpful to evaluate effusion cytology[1-10]. The

Table 1. Clinical diagnosis of the patients

Material	Number of samples	Clinical diagnosis
Pleural effusion	56	Primary lung cancer
(n = 108)	1	Malignant mesothelioma
	15	Metastatic pleural tumors (breast cancer, gastric cancer, mediastinal tumor, etc.)
	11	Pneumonia
	25	Other non-neoplastic diseases (empyema, pleuritis, etc.)
Peritoneal effusion	25	Primary ovarian tumor
(n = 74)	14	Endometrial carcinoma
	13	Other gynecological tumors (cervical cancer, peritoneal cancer, uterine myoma, etc.)
	17	Other non-gynecological tumors (hepatocellular carcinoma, pancreatic carcinoma, etc.)
	5	Non-neoplastic diseases (peritonitis, hepatic cirrhosis, liver abscess, etc.)

advantages of cell block are as follows: 1) prevention of artifacts, such as overcrowding of blood cell, inflammatory and epithelial cells, and drying of specimens[6]. 2) Possibility of storing slides in retrospective studies[6]. 3) Application of serial sections for special staining and immunostaining[1, 3, 4, 6]. 4) Suppression of nonspecific and unexpected immunoreaction often observed in immunocytochemistry[8]. 5) Evaluation of immunoreaction in the same cell clusters using serial sections. 6) Application to genetic analysis[11]. However, the disadvantage of cell block is the delay in diagnosis due to additional time for preparation[8]. Thus, several researchers stressed the overall advantage of cell block in cytological diagnosis[1-10]; however, no reports investigated the mismatched diagnosis between liquid-based cytology (LBC) and cell block. Thus, the aim of this study is to analyze the mismatched cases between LBC and cell block and to confirm the utility of cell block in improving diagnostic accuracy and quality in effusion cytology.

Materials and Methods

1. Materials

We used the cytological materials of 182 effusion samples (pleural effusion: 108 samples and peritoneal effusion: 74 samples) from September 2013 to January 2015 at the Department of Pathology, The Jikei University Hospital. The clinical diagnosis of the patients is shown in Table 1. For the 108 pleural effusion samples, primary lung cancer, malignant mesothelioma, and metastatic pleural tumors were suspected in 56, 1, and 15 samples, respectively. For the 74 peritoneal effusion samples, gynecological tumors and non-gynecological tumors were suspected in 52 and 17 samples respectively. More than one sample was often obtained from one patient. One hundred and eighty-two samples contained 143 cases of one sample, 13 cases of duplicate samples, three cases of triplicate samples, and one case of quadruplicate samples. For the immunostaining study of cell block, we used number of cases rather than number of samples. The number of cases in cell block study was, including two cases of duplicate samples. This study was approved by the Ethics Committee of the Jikei University School of Medicine for Biomedical Research. (29-036 (8652)).

2. Preparation of LBC specimens

The processing steps for the preparation of LBC slides were as follows: 1) the effusion was centrifuged at 3,000 rpm for two min and the supernatant was removed. 2) The pellet was suspended with the proper volume of Cyto Rich™ Red (catalog no. 491336) (Becton, Dickinson and Company, Franklin Lakes, NJ). The suspended sample was centrifuged at 3,000 rpm for two min and the supernatant was removed. 3) The pellet was resuspended with 1,000 μl distilled water, from which 500 μl mixture fluid was dropped on each of the two special slides (BD SurePth™ PreCoat Slides, catalog no. 491075) (Becton, Dickinson and Company). 4) The slides were left undisturbed for 10 min and then washed with 95% alcohol. One slide was stained with hematoxylin and eosin (HE) stain using Tissue-Tek Prisma (model: DRP-Prisma-JOD) (Sakura Finetek Japan CO., Ltd., Tokyo). Another slide was stained through periodic acid schiff (PAS) reaction by manual protocol.

3. Preparation of cell block

When the amount of sediment was more than the amount necessary to prepare the LBC specimen, a part of it was taken and processed to create a cell block. Cell blocks were prepared from residual samples after LBC

Table 2. Primary antibodies and experimental conditions

Primary antibody	Clone	Source	Dilution	Antigen retrieval
BCL2	124	DAKO	1:50	CC1 · Standard
BCL6	PG-B6p	DAKO	1:10	CC1 · Standard
CA125	M11	DAKO	1:100	None
CA19-9	116-NS-19-9	DAKO	1:10	None
Calretinin	Polyclonal (code No. 08-1211)	Invitrogen	Prediluted	CC1 · Standard
CD10	56C6	Leica	1:100	CC1 · Standard
CD20	L26	DAKO	1:50	CC1 · Standard
CD56	1B6	Nichirei	Prediluted	CC1 · Mild
CD79α	JCB117	DAKO	1:150	CC1 · Mild
CDX2	EPR2764Y	Nichirei	Prediluted	CC1 · Standard
CEA	II-7	DAKO	1:50	Protease · 4 min
Chromogranin A	DAK-A3	DAKO	1:200	CC1 · Standard
CK20	KS20.8	DAKO	1:100	Protease · 4 min
CK5/6	D5/16B4	DAKO	1:50	CC1 · Standard
CK7	OV-TL12/30	DAKO	1:100	Protease · 4 min
Cytokeratin (1/5/10/14)	34βE12	Leica	1:50	CC1 · Standard
D2-40	D2-40	DAKO	1:50	CC1 · Standard
ER	SP1	Roche	Prediluted	CC1 · Standard
GCDFP-15	23A3	Novocastra	1:120	CC1 · Mild
HER2	4B5	Roche	Prediluted	CC1 · Standard
Mammaglobin	304-1A5	DAKO	1:100	CC1 · Standard
Mesothelial cell	HBME-1	DAKO	1:50	Protease · 4 min
Napsin A	Polyclonal (Code: 418061)	Nichirei	Prediluted	CC1 · Standard
P40	Polyclonal (code: 418171)	Nichirei	1:100	CC1 · Standard
P53	Polyclonal (product code: NCL-L-p53-CM5p)	Leica	1:30	CC1 · Standard
P63	4A4	Nichirei	Prediluted	CC1 · Standard
PAX8	Polyclonal (catalog No. 10336-1-AP)	Proteintech	1:800	CC1 · Mild
PgR	1.00E+2	Roche	Prediluted	CC1 · Standard
Synaptophysin	27G12	Novocastra	1:50	CC1 · Short
TTF-1	8G7G3/1	DAKO	1:50	CC1 · Standard
WT-1	6F-H2	DAKO	1:50	CC1 · Mild

CC1, EDTA, PH 8.5, 100°C: CC2, Citric acid buffer, PH 6.0, 95°C
Short, 8 min: Mild, 30 min: Standard, 60 min
CEA, carcinoembryonic antigen: CK, cytokeratin; ER, estrogen receptor: HER2, human epidermal growth factor receptor 2: PgR, progesterone receptor: PSA, prostate specific antigen: TTF-1, thyroid transcription factor-1

preparation as follows: 1) the effusion was centrifuged in 1.5 ml microtube (PE sampling tube, catalog no. C33550-05) (ASIAKIZAI Co., Ltd., Tokyo) at 3,000 rpm for two min and the supernatant was removed. This microtube was soft and cut easily with blade. 2) Ten percent neutral buffered formalin was added slowly to the pellet in the microtube. The pellet was left undisturbed overnight for fixation. 3) The supernatant was removed and the tip of the microtube was cross-cut. The pellets in the microtubes were put in a plastic cassette and processed

using an automatic tissue processor, Tissue-Tek-VIP 6 (model: VIP6-JO) (Sakura Finetek Japan CO., Ltd.). 4) The pellets were taken out from the microtube and embedded in paraffin. 5) Each cell block was cut into 3 μm, and HE staining and PAS reaction were performed.

4. Immunostaining of cell block

The 3 μm-thick paraffin sections made from cell block were immunostained with Bench Mark XT Automated Slide Preparation System (model: 750-BXT) (Roche Diagnostics, K.K., Tokyo). The sources and experimental conditions of the antibodies are shown in Table 2. Histological types and primary sites of carcinoma were predicted with the results of immunostaining.

5. DNA analysis using cell block

Using two cell blocks of lung carcinoma, *ALK* rearrangement was examined by fluorescent in situ hybridization (FISH). Moreover, using four cell blocks of lung carcinoma, *EGFR* mutation was examined by real-time PCR. These molecular analyses were performed at SRL, Inc., Tokyo. FISH was performed using Vysis® *ALK* Break Apart FISH Probe kit (ABBOT JAPAN CO., LTD) . Real-time PCR was performed using Cobas® *EGFR* detecting mutation kit v2.0 (Roche Diagnostics, K.K., Tokyo).

6. Pathological evaluation

Cytological evaluation was classified as "negative", "suspicious", and "positive" by trained cytotechnologist and cytopathologist. Evaluation of cell block was classified as "benign", "atypical", and "malignant" by the pathologists. Diagnostic accuracy and concordance rate between LBC and cell block were calculated. In addition, we analyzed the difference between cytological and histological characteristics of LBC and cell block. Immunostaining was evaluated as positive when "positive" tumor cells exceeded 1%.

Results

1. Diagnostic difference between LBC and cell block

Comparison of the diagnosis between LBC and cell block is summarized in Table 3. Out of 182 samples, 98 samples were classified as negative in LBC and benign in cell block, and 79 samples were classified as suspicious or positive in LBC and atypical or malignant in cell block. Therefore, the diagnostic concordance rate was 97.3% (177/182 samples); however, five cases showed mismatched diagnosis between LBC and cell block. The review of the five cases is outlined in Table 4.

Table 3. Comparison of diagnosis between LBC and cell block

	Histological diagnosis of cell block		
LBC diagnosis	Benign	Atypical or malignant	Total
Negative	98	3	101
Suspicious or positive	2	79	81
Total	100	82	182

LBC, liquid-based cytology

The details of the five mismatched cases between LBC and cell block are as follows:

[Case 1] A 67 year-old male patient suspected to have primary lung cancer. Some mesothelial cells and non-atypical cells were observed in LBC. However, a few atypical cells with irregular nuclei were seen in cell block. These atypical cells were positive for TTF-1, but some reactive mesothelial cells were also positive for TTF-1 nonspecifically. We diagnosed Case 1 as atypical cells in the cell block, although it was difficult to distinguish the reactive mesothelial cells from neoplastic cells. A follow-up lung biopsy performed 10 days after the cytological examination was diagnosed as benign. Therefore, the cells diagnosed as atypical cells in cell block were probably reactive mesothelial cells with unignorable nuclear atypia. The patient died four months later due to lung cancer. [Case 2] A 64 year-old male patient with past history of rectal cancer. Metastatic lung cancer was clinically suspected. We observed some mesothelial cells and non-atypical cells in LBC (Figure 1). However, we observed a few clusters of atypical cells with high nuclear-cytoplasmic ratio and irregular nuclei in cell block. We diagnosed Case 2 as malignant (adenocarcinoma), because these atypical cells were positive for carcinoembryonic antigen (CEA). We reviewed LBC specimen; however, neoplastic cells resembling cells observed in cell block and surgical material were absent. We concluded that the neoplastic cells in the pleural effusion were present in small amounts, thus found only in cell block. The patient died one month later due to lung metastasis from rectal cancer. [Case 3] A 58 year-old female patient with cervical cancer. Some mesothelial cells and non-atypical cells were found in LBC (Figure 2). One cluster composed of epithelial cells with nuclear enlargement, hyperchromatic nuclei, and conspicuous nucleoli was observed in cell block. The cervical biopsy detected adenosquamous carcinoma six months before cytology. We diagnosed Case 3 as malignant (carcinoma) in cell block because these atypical cells appeared similar to cervical cancer morophologically. Neoplastic cells resembling cells obtained in cell block and biopsy was not seen in LBC. Neoplastic cells found in peritoneal

Table 4. Review of five cases with mismatched diagnosis between LBC and cell block

Case number	Effusion material	Age	Gender	LBC		Cell block		Predicted primary site of malignancy	Final pathological diagnosis	Clinical outcome
				Diagnosis	Amount of atypical or carcinoma cells	Diagnosis	Amount of atypical or carcinoma cells			
1	Pleura	67	Male	Negative	None	Atypical	A few	Not applicable	Benign lesion	Died four months later due to lung cancer
2	Pleura	64	Male	Negative	None	Malignant	A few	Rectum	Adenocarcinoma	Died one month later due to lung metastasis from rectal cancer
3	Peritoneum	58	Female	Negative	None	Malignant	A few	Uterine cervix	Carcinoma	Transferred six months later and lost to follow-up
4	Pleura	52	Male	Suspicious	A few	Benign	None	Not available	Not available	Lost to follow-up after three years
5	Pleura	68	Male	Suspicious	Several	Benign	None	Lung	Squamous cell carcinoma	Died three months later due to lung cancer

LBC, liquid-based cytology

effusion might be present only in cell block because of small amounts of malignant cells. The patient was transferred six months later and lost to follow-up. [Case 4] A 52 year-old male patient with hepatic cirrhosis. We diagnosed this case as suspicious in LBC, because a few atypical cells were present in PAS reaction, but not in Papanicolaou stain. We observed many inflammatory cells but a few mesothelial cells in cell block. A follow up histological examination was not performed, but 6-time repeated cytological examinations (once for pleural effusion and five times for peritoneal effusion) showed negative results except for this LBC. Therefore, we considered that a few atypical cells stained with PAS reaction were likely to be denatured mesothelial cells. The patient was lost to follow-up after three years. [Case 5] A 68 year-old male patient suspected to have primary lung cancer. The LBC of pleural effusion was diagnosed as suspicious, because we observed several irregularly overlapping cell clusters with irregularly shaped nucleus and increased chromatin. We could not deny the possibility of malignancy; however, we observed only inflammatory cells and mesothelial cells in cell block (Figure 3). The follow-up pulmonary biopsy performed on the next day after the cytological examination was diagnosed as squamous cell carcinoma. Pleural effusion cytology was performed for additional five times, but all the results were negative. The washing of endobronchial cytology was performed twice; the first was atypical cells and the second was negative. Therefore, the cells diagnosed as atypical cells in LBC were possibly reactive methothelial cells with moderate nuclear atypia, because these atypical cells did not look similar to the squamous cell carcinoma morphologically. The patient died three months later due to lung cancer.

Out of these five mismatched cases, four cases (Case 1–4) showed a few atypical cells only seen in LBC or in the cell block: Case 1–3 showed a few atypical cells only in cell block, and Case 4 showed a few atypical cells only in LBC, while Case 5 showed several atypical cells only in LBC.

2. Immunostaining results of cell block

The flowchart of the diagnosis in the 32 cases examined by immunostaining is outlined in Figure 4. Out of the 32 cases, two were diagnosed as benign cells, five as atypical cells, and 25 as malignant cells. The two benign cases showed non-atypical cells that were positive for mesothelial markers such as Calretinin, HBME1, and D2-40. Out of the 25 malignant cases, 22 cases showed carcinomas cells positive for epithelial markers such as CAM5.2, CK7, and CK20 and three cases showed B cell lymphoma cells positive for CD20 and CD79a. The 22 carcinoma cases included 17 cases of adenocarcinoma, three cases of small cell carcinoma, and two cases of carcinoma, not otherwise specified (NOS). Carcinomas,

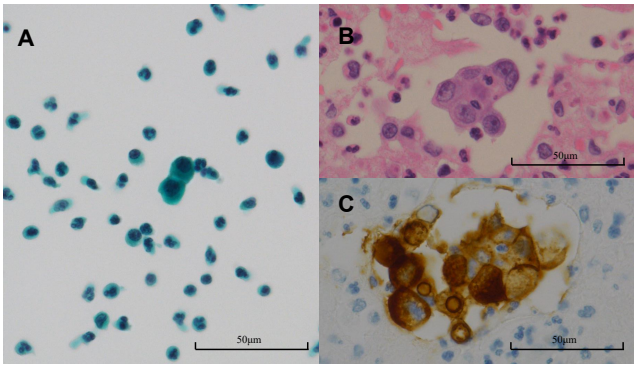

Figure 1. Case 2. Pleural effusion in a 64 year-old male patient

A: Papanicolaou stain in LBC (×400). Mesothelial cells and inflammatory cells are shown. B: HE stain and
C: Immunohistochemistry of carcinoembryonic antigen (CEA) in cell block (×400). The cluster of atypical cells with high N/C ratio are shown (B). These atypical cells are positive for CEA (C).

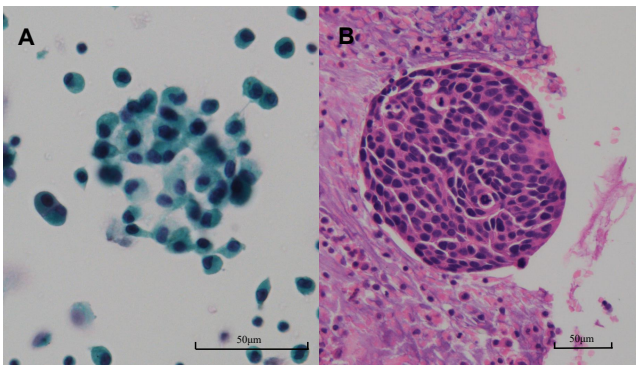

Figure 2. Case 3. Peritoneal effusion in a 58 year-old female patient

A: Papanicolaou stain in LBC (×400). Some mesothelial cells and non-atypical cells are shown. B: HE stain in cell block (×200). A streaming cluster composed of cells with nuclear enlargement and hyperchromatic nuclei is shown.

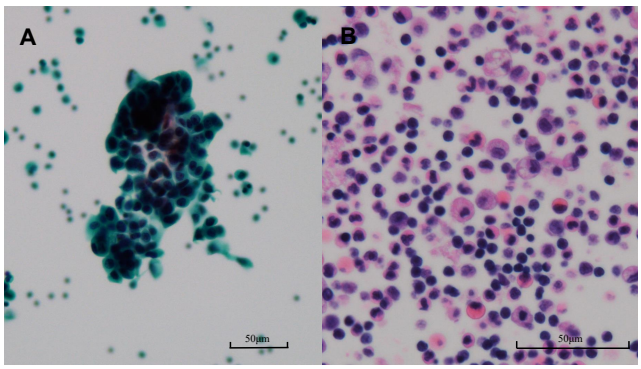

Figure 3. Case 5. Pleural effusion in a 68 year-old male patient

A: Papanicolaou stain in LBC (×200). Several overlapping cell clusters of atypical cells with irregular shapes of the nucleus and increased chromatin are shown. B: HE stain (×400). Many inflammatory cells are shown.

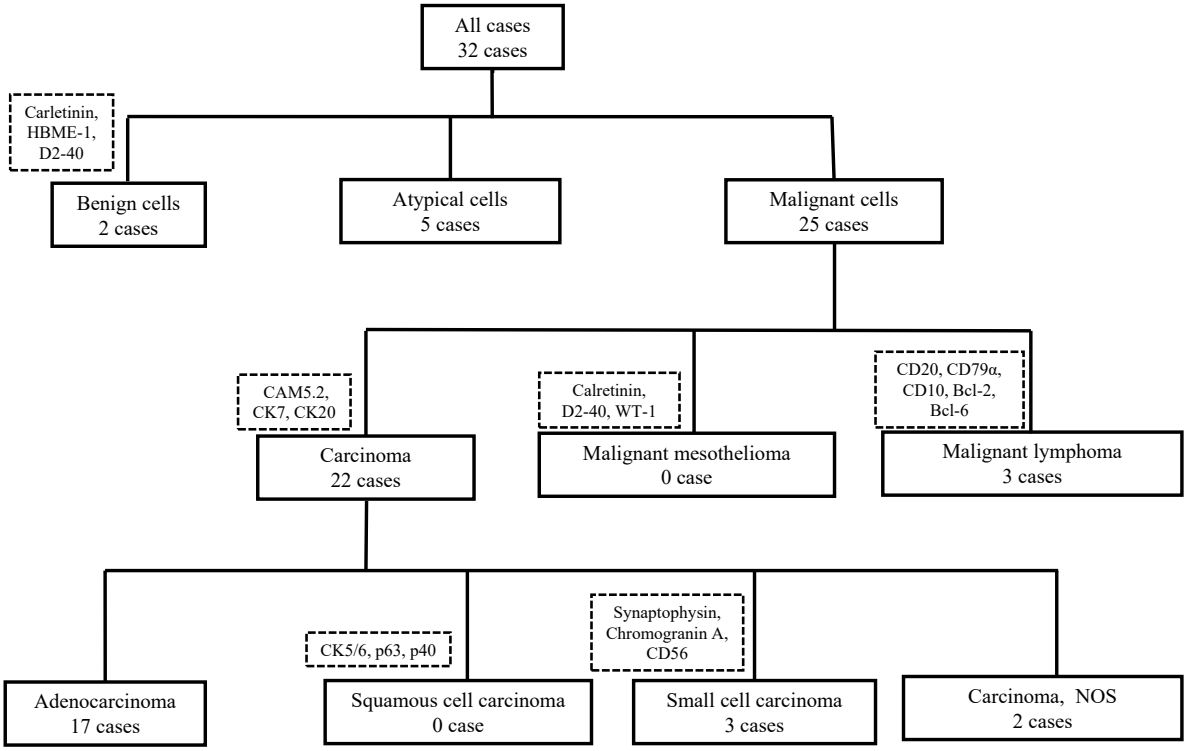

Figure 4. Flowchart of the 32 cases examined by immunostaining

Twenty-five malignant cases and two benign cases were distinguished by immunostaining using cell block. Furthermore, 17 adenocarcinomas and three small cell carcinomas were diagnosed among 22 carcinoma cases (91.0%).
Squares with dotted lines showed major useful antibodies for differential diagnosis.
NOS: not otherwise specified

NOS were diagnosed when the histology and immunostaining with mesothelial and epithelial biomarkers gave inconsistent results. Thus, histological types could be determined by immunostaining in 20 of the 22 carcinoma cases (91.0%). Adenocarcinomas were diagnosed using cell morphology and epithelial biomarkers. Small cell carcinomas were diagnosed using cell morphology and neuroendocrine markers such as synaptophysin, chromogranin A, and CD56.

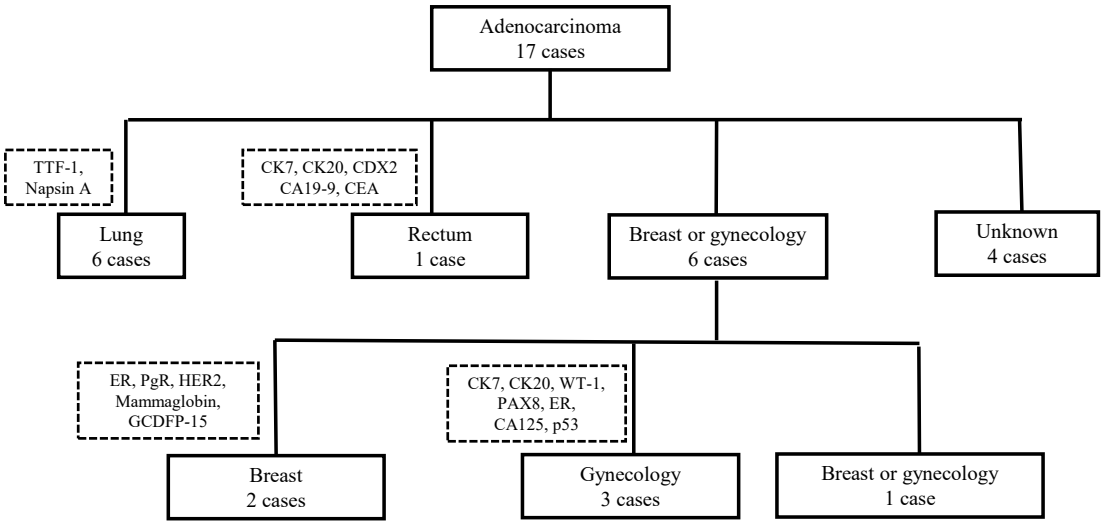

Figure 5. Flowchart of the evaluation of primary site in 17 adenocarcinomas examined by immunostaining

The primary sites were identified in 13 cases (76.5%) by immunostaining among 17 adenocarcinoma cases: six cases were diagnosed as metastasis from the lung, one case from the rectum, and six cases from the breast or gynecological organs.
Squares with dotted lines showed major useful antibodies for differential diagnosis

Furthermore, the flowchart of the diagnosis of primary site of 17 adenocarcinomas examined by immunostaining is outlined in Figure 5. Out of the 17 cases, six were diagnosed as metastasis from the lung due to TTF-1 positivity, one was from the rectum due to CDX2 positivity, two were from the breast due to reference to previous history and positivity of estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor (HER2), mammaglobin, and GCDFP-15, three were from gynecological organs, and one was from the breast or gynecological organs due to ER positivity without previous history. Origin could not be identified in four cases. As a result, primary sites could be identified in 13 cases among 17 adenocarcinomas cases by immunostaining (76.5%). Immunostaining could determine the application of hormone therapy or molecular targeted therapy to breast cancer. In the first case of breast cancer, the expressions of ER, PgR, and HER2 were positive. In the second case, immunostaining for ER was only performed and the expression was positive.

3. Results of DNA analysis using cell block

ALK rearrangement was examined by FISH in two cases of lung carcinoma, and *EGFR* mutation was examined by real-time PCR in four cases of lung carcinoma. The two cases were all negative for *ALK* rearrangement while *EGFR* mutation was positive only in two cases.

Discussion

In this study, the diagnostic concordance rate between LBC and cell block was 97.3%. Diagnosis was mismatched in five cases between LBC and cell block. The main cause of the mismatch was the small amount of atypical cells in LBC or cell block. Furthermore, immunostaining with cell block determined histological types in 91.0% of carcinomas, and predicted primary site in 76.5% of adenocarcinomas.

1. Comparison between LBC and cell block

Several studies have indicated the usefulness of cell block[1-10]. The combined use of cytology and cell block has also been reported, but the cytological preparation was done using conventional method in these reports[1, 2, 4, 6, 9]. The detection rate of malignancy ranges from 13% to 15% higher in cell block than conventional cytology[4, 6, 9]. The number of suspicious or positive cases are twice higher in combined use of cytology and cell block than cytology alone[1]. Khan, et al. reported that the primary site of cancer could be determined in 81% of carcinomas by the combined use of cytology and cell block, and the figures could increase to 90% by adding clinical information and radiological features[10].

LBC can prevent artifacts such as overlapping blood cells or inflammatory cells and drying of specimens.

Recently, LBC is used not only in gynecology but also in non-gynecologic field[12-14]. Gabridl, et al. showed that the detection rate of malignancy in effusion cytology improved by 6 % using LBC method compared to conventional method [15]. In this study, we indicated high diagnostic concordance rate between LBC and cell block (97.3%). However, the small amount of atypical cells in either sample found in this study, may lead to mismatched diagnosis.

2. Immunostaining of cell block

In effusion cytology, immunostaining is essential in determining histological types and predicting primary sites of carcinomas. From LBC method, we prepared extra samples from the residual samples and used them for special staining and immunostaining. In conventional immunocytochemistry, nonspecific or unexpected immunoreactivity is a major problem in effusion samples which contain protein-rich fluid such as mucin[16-17], and masking of antigen in immunostaining is often caused by fixation[11]. It is necessary to determine the optimal condition for the immunostaining, because the fixative solution of LBC is different between sample condition and commercial system. Kawahara, et al. evaluated that the frequency of immunoreactivity was lower on Cyto Rich™ Blue which was ethanol-based fixative than on Cyto Rich™ Red which was formalin-based fixative[11]. Hudock, et al. evaluated various fixative solutions for ER and PgR detection and demonstrated that cell blocks fixed in formalin solution yielded high frequency of positivity for both ER and PgR; however, ethanol-fixed materials, either smear or cell block, resulted in a significantly lower positivity[18]. Fetsch, et al. reported that the background of nonspecific reaction was seen in 66% of LBC (ThinPrep®, Hologic Japan, Inc, Tokyo) but in 17% of cell block[7]. Cell block is suitable for immunostaining of effusion specimens that have the morphology of malignant cells[7]. The staining condition for immunostaining using cell block is usually the same with that of immunohistochemistry, and can almost be used as the same for immunohistochemistry.

Furthermore, immunostaining of ER, PgR, and HER2 using cell block from pleural effusion could contribute to the diagnosis and treatment of brest cancer.

3. DNA analysis using cell block

Cell block could be used for DNA analysis with *ALK* rearrangement[19] and *EGFR* mutation[11]. In this study, we confirmed that cell block was useful for DNA analysis for molecular targeted treatment.

In conclusion, this study showed a high concordance rate between LBC and cell block in effusion cytology; however, the small amount of atypical cells in either samples may lead to mismatched diagnosis. We could often determine the histological types and predict the primary site of malignancy through cell block immunostaining, when a definitive diagnosis could not be reached with only morphology. Furthermore, cell block was also useful in DNA analysis. In recent years, the molecular targeted treatments have been remarkably developed. Therefore, preparation of cell block would become more useful for effective prediction of the molecular targeted treatment.

Acknowledgment: We are grateful to all staff of the Department of Pathology, Jikei University Hospital. AH designed the study, performed experiments and analyzed data, and drafted the manuscript; TU advised the presentation and edited the manuscript; MU and SI performed experiments; ST, TK, MI, HT edited the manuscript; SH, YS supervised the study and edited the manuscript; MS edited the manuscript and administered the project. All authors have read and approved the final manuscript.

Conflict of interest: The authors and their families declare that they have no conflict of interest.

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