Comparison of Immunocytochemical Sensitivity Between Formalin-Fixed and Alcohol-Fixed Specimens Reveals the Diagnostic Value of Alcohol-Fixed Cytocentrifuged Preparations in Malignant Effusion Cytology

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Abstract

The most commonly used fixative in effusion cytology is formalin. In the present study, the immunocytochemical properties of formalin-fixed and alcohol-fixed specimens were compared to evaluate the usefulness of alcohol-fixed cytocentrifuged preparations for routine cytologic diagnosis. A total of 269 effusion samples and 17 primary antibodies were used. The sensitivity of immunocytochemical studies in alcohol-fixed specimens was similar and correlated to that of formalin-fixed specimens, suggesting that alcohol-fixed cytocentrifuged preparations are useful in effusion cytology. Pretreatment with or without heat-induced antigen retrieval revealed that antigen retrieval was unnecessary for immunocytochemical studies with most primary antibodies in alcohol-fixed cytocentrifuged preparations. The present study describes the use of immunocytochemical studies with alcohol-fixed cytocentrifuged preparations for diagnosis in routine effusion cytology.

Histologic examinations of body cavity lesions are sometimes difficult. Effusion cytology is less aggressive for patients and easier than histologic examination. However, it is sometimes difficult to distinguish benign cells from malignant cells when malignant cells exhibit only slight atypia, reactive mesothelial (RM) cells show marked atypia, and there are few target cells. In addition, the identification of the primary organ in malignant effusions is difficult using only conventional cytomorphologic features. Therefore, immunocytochemical studies have a critical diagnostic role in effusion cytology. To date, numerous immunocytochemical analyses of effusion samples have been reported using various primary antibodies, including mesothelium-associated markers (calretinin, D2-40, cytokeratin [CK] 5/6, mesothelial cell [HBME-1], Wilms tumor 1 [WT1], adenocarcinoma-associated markers [epithelial antigen [Ber-EP4], epithelial-related antigen [MOC-31], carcinoembryonic antigen [CEA], and Leu-M1], and markers that distinguish neoplastic cells from RM cells (epithelial membrane antigen [EMA], IMP3, glucose transporter protein 1 [GLUT-1], and desmin). These antibodies are often used for routine examinations.

The most commonly used fixative for diagnosis and research is formalin, and many effusion studies use formalin-fixed, paraffin-embedded (FFPE) cell blocks (CBs). Formaldehyde acts as an intermolecular and intramolecular protein cross-linker. Heat-induced antigen retrieval (HIAR), which is used to disrupt the cross-links produced by fixation, is often carried out on FFPE sections and is a crucial procedure for immunohistochemical analysis. The HIAR technique is influenced by the fixative solution, fixation time, HIAR solution, pH of the HIAR solution, and cool-down time after heating. Therefore, the
standardization of immunohistochemical analysis for FFPE sections is difficult. On the other hand, alcohol, which is an organic solvent, fixes tissue in a nonadditive manner, and the preservation process does not require covalent bond formation between proteins. Instead, alcohol is thought to exert its effect by removing water molecules that are noncovalently bound to amino acid side chains. Treatment of most alcohol-fixed specimens with HIAR is therefore considered unnecessary. The alcohol-fixed Papanicolaou-stained specimen, which is prepared routinely in diagnostic cytology, is the best example of a sample that is not affected by the HIAR process.

Numerous immunocytochemical examinations using alcohol-fixed cytocentrifuged preparations (AFCPs) have been reported; however, to the best of our knowledge, no reports have compared the sensitivity of formalin-fixed and alcohol-fixed specimens in immunocytochemical studies. The present study compares FFPE and AFCP specimens from the same effusion sample using 17 commonly used primary antibodies and reveals the value of immunocytochemical studies using AFCP for effusion cytology.

Materials and Methods

Cases

Effusion specimens were obtained from a cohort of patients with malignant effusions. All malignant cases were confirmed by surgical biopsy or resection of the primary site. The diagnosis of diffuse malignant mesothelioma (DMM) was based on morphologic criteria and immunohistochemical staining and clinical findings. RM cells were derived from patients who had no clinical history of malignancy and no malignant cells identified in cytologic examinations. A total of 269 cases of pleural, peritoneal, and pericardial samples were evaluated. Effusion samples were processed as follows: (1) Effusion samples were centrifuged at 1,500 rpm for 5 minutes. (2) Sediment smears were fixed in 95% alcohol, stained using the Papanicolaou method, and then used for the cell transfer technique. (3) To the sediment that remained, 20% buffered formalin (pH 7.0) was added as a fixative for the CB sample.

The cell transfer technique for AFCPs was performed as follows: The slides were soaked in xylene to remove the coverslips and then covered with 1 mL of malinol diluted with an equal volume of xylene. These slides were then kept overnight at room temperature until the malinol coating was fixed. Next, the slides were soaked in a water bath at 37°C for 2 hours, and the softened malinol membrane was peeled off with forceps. The malinol membrane was cut into pieces with a scalpel blade, and each fragment was transferred to another glass slide in water. Then, the slides were dried for 1 hour at 60°C to ensure attachment. The slides were soaked in xylene to remove malinol, and immunocytochemical staining was performed.

To prepare FFPE-CB samples, the effusion was centrifuged at 1,500 rpm for 5 minutes in a pipette tip plugged with paraffin at the tip. Cell pellets were collected, and then dehydration and paraffin permeation were carried out with the same procedure used for routine surgical tissues. Finally, paraffin-embedded cell pellets inside the tip were cut into 3-μm-thick sections, and immunocytochemical staining was performed.

Immunocytochemical Analysis

Immunocytochemical analysis was performed using a highly sensitive indirect immunoperoxidase technique (Simple Stain MAX-PO MULTI, Nichirei, Tokyo, Japan). The indirect method is a 2-step method to detect antigens that uses an enzyme-labeled polymer secondary antibody. The primary antibodies used for this study were EMA, IMP3, GLUT-1, CEA, Ber-EP4, MOC-31, Leu-M1, calretinin, D2-40, HBME-1, WT1, CK5/6, desmin, thyroid transcription factor-1 (TTF-1), CA125, CK7, and CK20. The primary antibodies, clones, dilutions, pretreatments, and sources are listed in Table 1. For antigen retrieval by heat treatment, the sections were treated at 98°C for 20 or 40 minutes according to the manufacturer’s instructions and kept at room temperature for 20 minutes. For antigen retrieval by protease, the sections were incubated in 0.05% protease (type XXIV, bacterial; Sigma-Aldrich, St Louis, MO) for 30 minutes at room temperature.

FFPE sections of CBs were routinely deparaffinized in xylene, rehydrated in alcohol, and processed for pretreatment as follows: The sections were incubated in 3% hydrogen peroxide solution for 10 minutes to eliminate endogenous peroxidase activity and then incubated with 5% normal goat serum for 10 minutes at room temperature. Sections were then incubated with primary antibody overnight (18 hours) in a humid chamber followed by secondary antibody incubation for 30 minutes. Immunocomplexes of horseradish peroxidase were visualized by 3,3’-diaminobenzidine (DOJINDO, Kumamoto, Japan) reaction, and sections were counterstained with hematoxylin before mounting.

For the cytocentrifuged preparations, antigen retrieval was performed only for immunostains against IMP3, WT1, CK5/6, and TTF-1. For these primary antibodies, immunocytochemical studies were performed under both conditions, with and without antigen retrieval. Immunocytochemical staining for cytocentrifuged preparations was carried out in the same manner as for the CB sections. In addition, the same antigen-retrieval process used with the CB specimens was also used for staining. Malignant effusions of ovarian and lung adenocarcinoma were used for immunocytochemical studies of CA125 and TTF-1, respectively. When more than 10% of cells were positive, immunocytochemical results were considered positive.
Results

A total of 3,444 cases (pleural effusion, 1,425; ascites, 1,927; pericardial effusion, 92; men, 1,569; women, 1,875), including 2,561 (74.4%) negative cases, 145 (4.2%) false-positive cases, and 738 (21.4%) positive cases, were cytologically diagnosed in our hospital between January 2001 and December 2010. The primary foci among the 738 positive cases were lung (140 [19.0%]); stomach (106 [14.4%]); ovary (97 [13.1%]); uterus (43 [5.8%]); colon (34 [4.6%]); breast (23 [3.1%]); gallbladder, bile duct, and pancreas (21 [2.8%]); nodal lymphoma (17 [2.3%]); and other sites (48 [6.5%]). Histologic diagnosis was not made in 209 cases (28.3%). Histopathology in 534 malignant cases was adenocarcinoma (97%), small cell carcinoma (12% [2.2%]), squamous cell carcinoma (8% [1.5%]), DMM (11% [2.1%]), malignant lymphoma (30% [5.6%]), hepatocellular carcinoma (5% [0.9%]), and other type (36% [6.7%]). Among the 111 cases in which malignant cells were detected in cytocentrifuged preparations of the effusion cytology from 2008 to 2010 in our hospital, malignant cells were also observed in CB preparations in 95 cases (85.6%).

The comparisons of immunocytochemical results obtained with CB and cytocentrifuged specimens of RM cells, adenocarcinoma, and DMM are shown in Figure 1, Figure 2, and Figure 3, respectively. The data for EMA, IMP3, and GLUT-1 are derived from our previous examination. As shown in Figure 1, the immunocytochemical sensitivity in CB and cytocentrifuged specimens was comparable in 17 analyses in RM cells. The sensitivity of calretinin in the cytocentrifuged preparation (92%) was similar to that in CBs (96%) when only expression in the cytoplasm was evaluated as positive. The sensitivity was poor (26%) when only expression in the nucleus was evaluated as positive. In the CB preparation, immunocytochemical results of calretinin were evaluated as positive only when expression was observed in both the nucleus and cytoplasm.

The expression of WT1 was observed in 2% of RM cells in cytocentrifuged preparations. The sensitivity of WT1 in cytocentrifuged preparations increased to 45% when processed with HIAR, but the sensitivity in cytocentrifuged preparations was still lower than that in CBs (77%).

In the context of adenocarcinoma (Figure 2), no significant differences in immunocytochemical results were seen between CBs and cytocentrifuged preparations with most antibodies. The sensitivity of TTF-1 increased from 57% to 74% in cytocentrifuged preparations processed with HIAR, and the sensitivity in the processed specimens was similar to that in CBs (75%). The differences in the sensitivity of immunocytochemical studies with TTF-1 in lung adenocarcinoma demonstrate the effect of HIAR. No positive cells were observed without HIAR in AFCP (Image 1A), but after HIAR treatment, TTF-1+ nuclei were readily detected (Image 1B).

The analysis of DMM samples (Figure 3) showed no correlation between CB and cytocentrifuged preparations, probably owing to the inclusion of few CB samples. The expression profiles of calretinin and WT1 in DMM had characteristics similar to those of RM cells. The immunocytochemical sensitivity in AFCP of adenocarcinoma originating from various primary organs is shown in Figure 4, and Ber-EP4 and MOC-31 were identified as sensitive markers for the investigation of...
Figure 1: Comparison of immunocytochemical sensitivity between cell block and cytocentrifuged preparations in reactive mesothelial cells. * Inclusion of heat-induced antigen retrieval in cytocentrifuged preparations. Numbers in parentheses following the antibody name correspond to the cell block and cytocentrifuged number of cases examined. Ber-EP4, epithelial antigen; CEA, carcinoembryonic antigen; CK, cytokeratin; EMA, epithelial membrane antigen; GLUT-1, glucose transporter protein 1; HBME-1, mesothelial cell; MOC-31, epithelial-related antigen; TTF-1, thyroid transcription factor 1; WT1, Wilms tumor 1. Calretinin, Cy and Calretinin, N refer to cytoplasmic and nuclear positivity in cytocentrifuged preparations, respectively.

Figure 2: Comparison of immunocytochemical sensitivity between cell block and cytocentrifuged preparations in adenocarcinoma. * Inclusion of heat-induced antigen retrieval in cytocentrifuged preparations. Numbers in parentheses following the antibody name correspond to the cell block and cytocentrifuged number of cases examined. Ber-EP4, epithelial antigen; CEA, carcinoembryonic antigen; CK, cytokeratin; EMA, epithelial membrane antigen; GLUT-1, glucose transporter protein 1; HBME-1, mesothelial cell; MOC-31, epithelial-related antigen; TTF-1, thyroid transcription factor 1; WT1, Wilms tumor 1. Calretinin, Cy and Calretinin, N refer to cytoplasmic and nuclear positivity in cytocentrifuged preparations, respectively.
Figure 3. Comparison of immunocytochemical sensitivity between cell block and cytocentrifuged preparations in diffuse malignant mesothelioma. * Inclusion of the heat-induced antigen retrieval in cytocentrifuged preparations. Numbers in parentheses following the antibody name correspond to the cell block and cytocentrifuged number of cases examined. Ber-EP4, epithelial antigen; CEA, carcinoembryonic antigen; CK, cytokeratin; EMA, epithelial membrane antigen; GLUT-1, glucose transporter protein 1; HBME-1, mesothelial cell; MOC-31, epithelial-related antigen; TTF-1, thyroid transcription factor 1; WT1, Wilms tumor 1. Calretinin, Cy and Calretinin, N refer to cytoplasmic and nuclear positivity in cytocentrifuged preparations, respectively.

Figure 4. Comparison of the immunocytochemical sensitivity between cytocentrifuged preparations of adenocarcinomas originating in different primary organs. Ber-EP4, epithelial antigen; CBD, common bile duct; CEA, carcinoembryonic antigen; GB, gallbladder; MOC-31, epithelial-related antigen; Panc, pancreas.

Image 1. Representative features of immunocytochemical thyroid transcription factor (TTF)-1 staining of the cytocentrifuged preparation in the same sample. A. Lung adenocarcinoma cells without antigen retrieval are negative for TTF-1 (×400). B. The antigen-retrieved nuclei of lung adenocarcinoma cells are positive for TTF-1 (×400).
primary organs. In addition, CEA was a sensitive marker for gastric cancer.

Staining comparisons of CB and cytocentrifuged preparations are shown in Table 2. A total of 9 primary antibodies, including EMA, GLUT-1, Ber-EP4, CEA, Leu-M1, calretinin, D2-40, HBME-1, and desmin, showed a significant correlation between the CB and cytocentrifuged preparations. The calretinin stain showed a correlation between the 2 preparations only when cytoplasm positivity was included. A correlation was present in stains for IMP3, CK5/6, and TTF-1 when processed with HIAR, but no correlation was detected in the WT1 stain between the CB and cytocentrifuged preparations. It is interesting that in the MOC-31 stain, 12 cases were FFPE-CB–/AFCP+, and 11 cases among them were adenocarcinoma. The main difference between FFPE-CB and AFCP is shown in Image 2. The expression of MOC-31 in adenocarcinoma was negative in FFPE-CB (Image 2A), but positive in AFCP (Image 2B), indicating that the sensitivity of AFCP is superior to that of FFPE-CB in some cases.

The distinction between benign and malignant cells is an important issue in effusion cytology, as is the determination of the origin of malignant cells. To address this question, schematic data for immunocytochemical studies are summarized in Figure 5. Immunocytochemical sensitivities are indicated in parentheses under the antibody name. For example, “calretinin (2%:92%)” indicates that calretinin is 2% positive in adenocarcinoma and 92% positive in RM cells.

Discussion

The appearance of neoplastic cells in an effusion is an important factor in determining prognosis and treatment.
Histopathologic examinations are sometimes difficult in body cavities. In addition, it is not uncommon for a malignant effusion to be the first clinical manifestation of undetected cancer. Thus, accuracy in the diagnostic cytology of effusions is critical. Among 738 malignant effusions included in the present study, the primary organs of malignancy were unknown in 28.3% of cases, and DMM, which is necessary to distinguish adenocarcinoma, existed in 2.1% of cases, demonstrating the critical role of immunocytochemical studies in achieving a correct diagnosis.

In effusion cytology, several reports of immunocytochemical analyses using FFPE-CB have been published, but no reports describe the practical use of AFCP in immunocytochemical studies. The present study shows that the sensitivity of immunocytochemical studies using AFCP for the detection of malignant cells was comparable to that of immunocytochemical studies of CB.

In FFPE-CB, immunocytochemical analysis of the effusion sample depends on cell volume. In contrast, immunocytochemical analysis using AFCP is independent of cell volume. Even if the AFCP contains limited cell volumes, recent advances in the cell transfer technique make it possible to perform immunocytochemical analysis using several antibodies.

At present, manufacturer’s instructions show immunohistochemical data that pertain to the analysis of formalin-fixed but not alcohol-fixed samples. To address whether immunocytochemical studies of AFCP are applicable to routine effusion cytology, we studied numerous effusions and found a significant correlation between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly correlated between FFPE-CB and desmin, was significantly cor

Since its development in 1991 by Shi et al., HIAR has become the most popular technique in routine immunohistochemical analysis. Several heating methods have been applied to HIAR such as microwave, conventional heating by water bath or hot plate, autoclave, electric pot, or domestic pressure cooker and steamer. HIAR has increasingly been used in pathology and analytic morphologic studies, and it has been applied in a wide range of clinical and research projects. Although the disruption of formaldehyde-induced cross-links is believed to be important, the mechanisms of HIAR are not fully understood.

Yamashita and Okada reported that pH is an essential factor for the proper refolding of epitopes. Reports in the literature demonstrate that the fixative solution, fixation time, pH of the HIAR solution, and cool-down time after heating influence the staining results. HIAR has generally been considered unnecessary for alcohol-fixed specimens, but the effects of HIAR in alcohol-fixed specimens have been reported. In the present study, the sensitivity of 9 primary antibodies, including EMA, GLUT-1, Ber-EP4, CEA, Leu-M1, calretinin, D2-40, HBME-1, and desmin, was significantly correlated between FFPE-CB and cytocentrifuged preparations even without the application of antigen-retrieval methods. Based on the present data and the cell loss that occurs as a consequence of HIAR treatment of cytocentrifuged preparations, in addition to the difficulty of counterstaining sections with hematoxylin, we suggest that processing cytocentrifuged preparations without HIAR is a more suitable method for these antibodies.

In contrast, the sensitivity of immunocytochemical studies against IMP3, WT1, CK5/6, and TTF-1 in AFCP showed better correlation with that in FFPE samples when HIAR treatment was included. This finding indicates that HIAR not only disrupts formaldehyde-induced cross-links but also may have a role in epitope presentation. In particular, detection of nuclear antigens required HIAR, consistent with the findings of previous studies. Slyaden et al. examined the estrogen receptor in frozen sections and suggested that microwaving changes only the extractability of the estrogen receptor, presumably by stabilizing the entire complex of...
Kawai et al used heat to denature double-stranded DNA into single-stranded DNA and suggested that this might allow the antibody access to a DNA binding protein whose antigenicity was partly hindered by steric effects. We also suggest that heat treatment in alcohol-fixed specimens is necessary for the detection of nuclear antigens.

In immunocytochemical studies of calretinin, the HIAR process was found to be unnecessary for alcohol-fixed specimens. The detection of calretinin showed good sensitivity without HIAR, and the sensitivity of immunocytochemical studies in AFCP was similar to that in CB when cytoplasm-positive cells were included (Figure 1; Table 2). In contrast with calretinin, the sensitivity of immunocytochemical studies in AFCP with an antibody against WT1, a nuclear antigen, increased with HIAR, but the sensitivity was still lower than that in FFPE sections (Figure 1; Table 2). The reason for this difference is not clear, but it is possible that the antigenicity declined and/or was eluted. WT1 staining in alcohol-fixed specimens is therefore not useful because of its low sensitivity. Although IMP3 is a cytoplasmic antigen, the sensitivity of IMP3 in HIAR-treated AFCP correlated exceptionally well with that in FFPE-CB (Table 2), suggesting the possibility that protein degeneration caused by heating could have resulted in loss of the antigen.

HBME-1 and WT1, which are mesothelial markers, were positive in adenocarcinoma, consistent with the findings of previous studies. In ovarian cancer, the positivity rates of HBME-1 and WT1 were 69.0% and 66.7%, respectively. It should be noted that CA125, which is used as an ovarian cancer marker, was also positive in mesothelial cells. WT1 and CA125 are useful in identifying the primary focus in combination with cytomorphic features.

CK and desmin, markers of intermediate filaments, showed higher sensitivity in immunocytochemical studies of AFCPs than in FFPE sections (Figures 1 and 2). This result suggests that alcohol fixation preserves antigenicity better than formalin fixation in certain cases and that alcohol fixation is suitable for the detection of intermediate filaments.

In MOC-31 staining, adenocarcinoma in 11 of 12 cases was FFPE-CB+/AFCP+. The sensitivity rates of MOC-31 were 70% and 85% in FFPE-CB and AFCP, respectively. These results showed that AFCP does not produce false-positives and that AFCP is more useful than FFPE-CB for the immunocytochemical studies of MOC-31.

In the CK7, CK20, CA125, and mesothelioma studies, no correlation was observed between formalin-fixed and alcohol-fixed specimens. However, this lack of correlation could be attributed to the small number of cases included in this examination or to the difference in cell volume between CB and cytocentrifuged preparations.

Immunocytochemical studies in AFCP showed good sensitivity, and the results obtained correlated with those of immunocytochemical studies in FFPE-CB. In a few cases, the sensitivity in AFCP was better than that in FFPE-CB. Antigen retrieval was not necessary for AFCP with most primary antibodies. The combinations of the primary antibodies Ber-EP4 and MOC-31 for adenocarcinoma, calretinin and D2-40 for mesothelial cells, and EMA, IMP3, and GLUT-1 for malignant cells were useful for diagnosis in effusion cytology. The sensitivity of immunocytochemical studies under HIAR conditions differed among the primary antibodies, suggesting that the assessment of the characteristics of each primary antibody is important, including antibodies specific against nuclear antigens and cytoplasmic or cell surface antigens. Our results provide evidence that the use of AFCP is valuable for routine immunocytochemical examinations in effusion cytology, and we think that the performance of immunocytochemical studies in cytologic diagnosis could be improved by combining it with destaining and cell transfer techniques.

References


