Original Article

Immunoreactivity of TTF-1, GATA-3, CEA, and p16/Ki67 cocktail in Cellprep®- processed control samples : Comparison of long-term storage in vials and slides

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Short Title: Immunoreactivity in Cellprep®- processed control samples

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Number of Tables: 2

Number of Figures: 3

Abstract

Introduction:

Cellprep® (CP) is a novel liquid-based cytology (LBC) system. This study aimed to assess the immunoreactivity of control samples stored long-term under two storage conditions using CP.

Methods:

The immunoreactivity in control samples was evaluated under two sample storage conditions: CP vial storage at room temperature (RT: 20–25 ℃) and CP slide storage in a refrigerator (2–6 ℃). Clinical samples as controls (total: positive/negative) were immunostained using thyroid transcription factor-1 (TTF-1) (20: 14/6), GATA binding protein 3 (GATA-3) (13: 10/3), carcinoembryonic antigen (CEA) (23: 15/8), and the p16/Ki67 cocktail (20: 12/8) markers. The first immunocytochemistry (ICC) was performed within 1 month using CP vials stored at RT. Samples stored in CP vials and on CP slides were used for the second (within 3–6 months) and third (within 6–11 months) ICCs. Compared with the first ICC, the concordance of immunoreactivity for the second and third ICCs was evaluated using the weighted kappa coefficient.

Results:

For TTF-1, CEA, and the p16/Ki67 cocktail markers, ICC controls had stable immunoreactivity for a minimum of 6 months when samples were stored in CP vials (kappa coefficients > 0.8), whereas for GATA-3, they were for 3 months. On CP slides, only for the p16/Ki67 cocktail, ICC controls had stable immunoreactivity for at least 3 months (kappa coefficient > 0.8).

Conclusion:

Clinical samples as ICC controls revealed consistently more stable immunoreactivity in CP vials than on CP slides for TTF-1, GATA-3, CEA, and the p16/Ki67 cocktail markers.

Keywords: immunocytochemistry, liquid-based cytology, control, storage, Cellprep, vial, slide

Introduction

Liquid-based cytology (LBC) has made it possible to preserve cellular components in the sample. Therefore, after a Pap test has been performed, additional slide preparations are available for ancillary tests such as immunocytochemistry (ICC) and molecular analysis as needed. In recent years, molecular analysis using LBC has extended beyond human papillomavirus DNA [1] to include analyses of driver gene mutations,[2] microRNAs,[3] and cancer genome profiling using next-generation sequencing.[4] Among these tests, ICC provides important diagnostic insights when biopsy samples are difficult to obtain or when the sample is fluid. Moreover, ICC can be used to assess tissue subtypes and predictive markers and distinguish primary lesions from metastatic lesions.[5] Specifically, it has been reported to help identify non-small cell lung carcinoma and neuroendocrine tumors in pulmonary samples.[6] Additionally, ICC can be used to screen for the expression of ALK, ROS1, and programmed death ligand-1 (PDL-1).[5] In effusion samples, it has been used to distinguish between reactive mesothelial cells, metastatic carcinomas, and malignant mesothelioma and to identify the primary lesion.[6] In gynecological samples, p16/Ki67 dual staining has been reported to be useful during the triage of high-risk HPV-positive women for the detection of high-grade cervical intraepithelial lesions.[7] The p16/Ki67 dual stain, marketed as CINtec® *PLUS* Cytology (CINtec; Roche Diagnostics), is the only commercially available test using a dual-biomarker for ICC. Using this established dual stain, we have previously demonstrated the utility of CINtec in detecting cervical adenocarcinoma, which is not sufficiently detectable by the Pap test alone.[8]

Cell blocks allow the immunostaining of samples using the same protocol as formalin-fixed and paraffin-embedded (FFPE) sections but require an adequate sample volume to prepare. However, in clinical practice, the sample volumes are often limited.[2] LBC is useful for preparing slides with limited sample volumes. The ICC process for slides prepared using alcohol-based fixation must be revalidated at each laboratory with reference to the immunohistochemistry (IHC) protocol because the immunostaining protocol was originally intended for IHC on FFPE sections. Therefore, using an appropriate ICC control is essential.

Cellprep® (CP) (Roche Diagnostics) is a novel LBC system compared with ThinPrep® (Hologic) and SurePath™ (Becton-Dickinson). The cells collected in the CP vials are agitated using wind pressure, collected onto an externally mounted filter with negative pressure, and smeared onto a slide glass.[9] As a result, cells are not subject to excessive strain. In this study, the immunoreactivity of clinical samples stored long-term for ICC controls using CP was investigated with a focus on two storage conditions: CP vial storage at room temperature (RT: 20–25 ℃) and CP slide storage in the refrigerator (2–6 ℃).

Materials and Methods

Thyroid transcription factor-1 (TTF-1; nuclear antigen), GATA binding protein 3 (GATA-3; nuclear antigen), carcinoembryonic antigen (CEA; cell membrane antigen), and p16/Ki67 cocktail (nuclear and cytoplasmic antigen), which were frequently used markers in our laboratory, were used as primary antibodies in the current study.

After sample collection, samples were immediately stored in CP vials (Cellprep® LBC Solution - Cervical, Oral Cavity for cervical samples; and Cellprep® LBC Solution - Body Fluid, FNA for other samples). The CP vials for the Pap test were stored overnight at RT, and the slides were subsequently prepared using the Cellprep® PLUS device (Roche Diagnostics, Tokyo, Japan). The sufficiency of cell numbers and the presence or absence of tumor cells were confirmed by the Pap test, and samples stored in the CP vials were divided into positive and negative control samples.

Twenty samples for TTF-1, 13 for GATA-3, 23 for CEA, and 20 for the p16/Ki67 cocktail markers were analyzed. Positive controls of ICC for TTF-1 were used in 14 samples containing adenocarcinoma cells of lung origin; the negative controls comprised 6 samples. Positive controls of ICC for GATA-3 were used in 10 samples containing malignant cells of breast origin; the negative controls comprised 3 samples. Positive controls of ICC for CEA were used in 15 samples containing adenocarcinoma cells (origin; stomach 8, pancreas 6, bile duct 1); the negative controls comprised 8 samples. Positive controls of ICC for the p16/Ki67 cocktail were used in 12 samples containing tumor cells of uterine cervix origin; the negative controls comprised 8 samples. Body cavity fluids, fine needle aspirates, and scrapings of lesions were used as samples.

Using residual samples stored in CP vials at RT, three slides for each sample (the first ICC, the second, and third ICCs as CP slide storage) were prepared within 1 month (1–31 days; mean 11 days, median 11 days) after sample collection in CP vials, and post-fixed with 95% ethanol for 15–19 h. The first ICC was performed immediately.

The second and third ICCs were performed using samples stored under two different conditions. In the first condition, samples were stored in CP vials at RT and prepared the day before performing ICC, post-fixed with 95% ethanol for 15–19 h. In the other, CP slides for the second and third ICCs were prepared at the same time as that for the first ICC, post-fixed with 95% ethanol for 15–19 h, air-dried, and stored in a slide box for refrigeration until ICC was performed. All samples were prepared using a Cellprep® Pre-coated Slide (Roche Diagnostics, Tokyo, Japan).

After sample collection in CP vials, samples were stored for 3–6 months (range: 95–172 days, mean: 131 days, median: 135 days) until the second ICC and 6–11 months (range: 204–311 days, mean: 255 days, median: 256 days) until the third ICC. The interval between the first and second ICCs

was 3–5 months (range: 92–144 days, mean: 120 days, median: 121 days), and that between the second and third ICCs was 3–6 months (range: 92–165 days, mean: 124 days, median: 121 days). Subsequently, the second and third ICCs were performed using the same protocol as the first ICC. The second and third ICCs of paired CP vials and CP slides for the same sample were performed on the same day (Figure 1). In addition, multiple clinical samples collected at different time points were run simultaneously on an automated immunostainer.

The ICC was performed using VENTANA BenchMark GX (Roche Diagnostics, Tokyo, Japan) and the VENTANA OptiView DAB detection system for TTF-1 and GATA-3, VENTANA I-View DAB detection system for CEA, and CINtec® *PLUS* Cytology Kit (Roche Diagnostics, Tokyo, Japan) for the p16/Ki67 cocktail. In the ICC for TTF-1, GATA-3, and CEA, samples were incubated with one drop (100 µl) of primary antibody for 8 min without deparaffinization. The ICC procedure for the p16/Ki67 cocktail followed the ThinPrep® specification in the manufacturer's instructions. As primary antibodies, anti-TTF-1 (SP141) Rabbit Monoclonal Antibody (Roche), anti-GATA-3 (L50-823) Mouse Monoclonal Antibody (Roche), anti-CEA (CEA31) Mouse Monoclonal Antibody (Roche), and anti-p16/Ki67 (p16; E6H4^{TM}/ Ki67; 274-11 AC3) Mouse/ Rabbit Monoclonal Antibodies (Roche) were used. The epitope retrieval of ICCs for TTF-1 and GATA-3 was performed at 95°C for 8 minutes with a CC1 retrieval solution (Ventana), and that of the ICC for the p16/Ki67 cocktail was performed at 99°C for 16 minutes with a CC1 retrieval solution.

The ICC scores for TTF-1, GATA-3, CEA, and the p16/Ki67 cocktail were determined according to the classifications shown in Table 1. The applicability of the ICC controls for TTF-1, GATA-3, and CEA was evaluated using the total score (TS) of the proportion of positive cells (proportion score; PS) and staining intensity (intensity score; IS), with reference to the Allred score used to assess hormone receptors in breast cancer.[10] In this study, PS was defined as the ratio of positive cells to the total number of cells. Regarding the determination of specimens with weakened staining intensity, specimens close to IS 3 (strong) were regarded as IS 2 (intermediate), and those close to IS 0 (no cells stained) were regarded as IS 1 (weak). The IS was scored for predominant positive cells in the whole slide. p16/Ki67 dual staining was considered “positive” only if cytoplasmic and/or nuclear (brown) staining for p16 and nuclear (red) staining for Ki67 were observed within the same cell. Weak positive staining for either p16 or Ki67 or both was considered “weakly positive”. Specifically, specimens with uneven or incomplete stainability were determined to be "weakly positive". The absence of staining for either p16 or Ki67 or both was considered “negative”. Three investigators (A.R., Y.U. and S.N.) were involved in the scoring. The scoring was performed with reference to the first ICC specimen without considering vial or slide storage, and storage period. The ICC scores for TTF-1, GATA-3, CEA, and the p16/Ki67 cocktail were performed individually by two investigators (A.R. and Y.U., or A.R.

and S.N.). If the score was inconsistent between two investigators, the final score was determined after discussion and re-scoring.

In samples of ICC controls for TTF-1, GATA-3, CEA, and the p16/Ki67 cocktail, in CP vials stored at RT and on CP slides stored in the refrigerator, the scores for the second and third ICCs were compared with the scores of the first ICC. In comparisons of paired CP vial and CP slide storage, differences in immunoreactivity were defined as a difference of ≥2 points in each score. As regards the p16/Ki67 cocktail, samples with a positive status changed to weak or negative statuses were considered attenuated.

Statistical analysis

The first ICC scores were compared with the second and third ICC scores obtained from samples stored in CP vials and on CP slides. Compared with the first ICC score, the concordance of immunoreactivity for the second and third ICC scores was evaluated using weighted Cohen's kappa. The weighted Cohen's kappa was calculated using the "irr" package in R version 4.2.1 (2022) with the function kappa2 (“equal").[11] A kappa value > 0.6 indicates substantial agreement, and a kappa value > 0.8 indicates almost perfect agreement.[12] In this study, a kappa value of > 0.8 was used to define the acceptability of the ICC control because scores of samples used for quality control must closely agree with the first ICC score.

Statement of Ethics

This study protocol was reviewed and approved by the Ethics Committee of Osaka International Cancer Institute, approval number 22155. Opt-out was performed as an alternative to written informed consent with the approval of the Ethics Committee of Osaka International Cancer Institute (approval number 22155) and according to the ethical guidelines of the Ministry of Health, Labor and Welfare.

Results

Immunostaining concordance in the second and third ICC scores compared with the first ICC score was assessed using the weighted kappa statistic. Of the samples stored in CP vials at RT, the second ICC scores for TTF-1, GATA-3, CEA, and the p16/Ki67 cocktail markers, and the third ICC scores for TTF-1, CEA, and the p16/Ki67 cocktail markers had kappa coefficients > 0.8. Of the samples stored on

CP slides in the refrigerator, only the second ICC score for the p16/Ki67 cocktail had a kappa coefficient > 0.8 (Table 2, Figure 2-3).

Comparing paired CP vial and CP slide storage, the difference in immunoreactivity was investigated with each antibody. Of positive controls, in the second ICC, three samples (3/14; 21%) for TTF-1, seven samples (7/10; 70%) for GATA-3, 11 samples (11/15; 73%) for CEA, and one sample (1/12; 8%) for the p16/Ki67 cocktail presented differences in immunoreactivity. In the third ICC, seven samples (7/14; 50%) for TTF-1, 10 samples (10/10; 100%) for GATA-3, 14 samples (14/15; 93%) for CEA, and four samples (4/12; 33%) for the p16/Ki67 cocktail also revealed differences in immunoreactivity. Except for one sample of GATA-3 in the third ICC, these differences were due to reduced immunoreactivity after CP slide storage.

In the negative controls for TTF-1, GATA-3, CEA, and the p16/Ki67 cocktail, no increase in immunoreactivity was observed in samples stored in CP vials or on CP slides during these storage periods.

Discussion

ICC has become an essential adjunct for confirming diagnoses and evaluating biomarkers when only cytological samples are available. However, in contrast to IHC, ICC quality control has been left behind.[13] In this study, storage conditions and storage periods suitable for the ICC control on LBC samples by CP were investigated. We evaluated the immunoreactivity for TTF-1, GATA-3, and CEA according to characteristic criteria with reference to the Allred score,[10] which considers the proportion and intensity scores. The attenuation of staining was defined as a decrease of ≥ 2 scores from the first score. Because a difference of ≥ 2 scores was a decrease in both IS and PS or a decrease of ≥ 2 scores in either PS or IS, it was more objective than a one-score difference. The score was simplified as the evaluation of immunoreactivity for the p16/Ki67 cocktail was based on double staining.

The immunoreactivity for TTF-1, GATA-3, and CEA markers yielded better results for samples stored in CP vials at RT than for those stored on CP slides under refrigeration. Only for the p16/Ki67 cocktail, the stainability of samples was approximately similar in samples stored in CP vials at RT and on CP slides under refrigeration within at least 3 months after sample collection. Samples stored in the CP vials were considered applicable as the ICC control, with a minimum storage period of 3 months. The ICC controls for TTF-1, CEA, and the p16/Ki67 cocktail also had stable immunoreactivity for at least 6 months. The storage of control samples at RT facilitates the management of the storage period and remaining samples. Recognition of the applicable storage periods is useful for collecting

controls. However, there were some differences in individual immunoreactivity and degree of attenuation in each clinical sample. This should be considered when clinical samples are used as controls.

To date, there have been several reports on the storage conditions of LBC samples for the ICC quality control, analyzed using cell lines [14-17] or clinical samples.[9, 18, 19] However, these studies used LBC samples processed with ThinPrep® [14-16, 18] or SurePath™.[17, 19] We previously used LBC samples prepared using CP to examine the storage period of samples for the ICC in detecting estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2).[9] There are no publications specific to ICC controls in LBC samples using CP. This study is the first to investigate in detail storage conditions suitable for the ICC in LBC samples prepared with CP.

According to previous reports, LBC samples were stored either in a preservation solution or on a slide. Moreover, the storage temperature was set to RT for samples stored in preservation solution, [9, 14, 16, 17] and various temperatures for samples stored on slides.[14, 15, 18, 19]

Zappacosta R et al. investigated whether Thinprep®-processed samples stored in PreservCyt solution at RT could detect antigens after a long storage period. As a result, phosphorylation of the nuclear transcription factor Stat-5 (pStat-5) was detected for up to four months, and nuclear positivity for the proliferation index MIB-1 and membrane positivity for the CD30 antigen was evident for three months.[16] Sato H et al. examined the immunoreactivity for CD20, leukocyte common antigen (LCA), and Ki67 using SurePath™ -processed samples stored in Cytorich red ™ and Cytorich blue ™ solution at RT for a specified period. According to their results, the positivity rate for CD20 and LCA was still maintained in samples stored for 6 months, whereas the Ki67 positivity rate was highest at 1 hour and markedly decreased with time.[17] The type of LBC system and the location of the antigen should be considered for the ICC of samples stored in preservation solution at RT.

In our study, CP slides post-fixed in 95% ethanol, air-dried and refrigerated did not maintain immunoreactivity to TTF-1, GATA-3, or CEA when stored for more than 3 months, whereas it was maintained for the p16/Ki67 cocktail. The dual stain kit by anti-p16/Ki67 cocktail is a commercial product. The reproducible immunoreactivity for anti-p16/Ki67 cocktail may be due to established protocols. The decrease of ICC scores for anti-TTF-1, GATA-3, or CEA could be attributed to loss of stainability caused by the ICC protocols.

Sauer et al. examined the immunoreactivity of ER and progesterone receptor (PgR) on slides, which were post-fixed in 4% formalin after long-term storage at -20 and -74 °C on slides prepared from liquid-based samples. They concluded that there was no significant loss of immunoreactivity for at least 6 months.[18] Additionally, Wiatrowska et al. demonstrated that ThinPrep®-processed slides stored at -70 °C and the air-dried cytospins stored at -70 °C retained immunoreactivity for at least 3

months for multiple primary antibodies. The ThinPrep®-processed slides used in their study were immediately fixed with 95% alcohol, post-fixed with Sprayfix®, and air-dried.[15] However, the study reported that ThinPrep®-processed slides, which 1) fixed in 95% ethanol only and stored at -70 °C or 2) post-fixed by Sprayfix® and stored at RT, had poor results for most tested antibodies. Tabbara et al. reported that ThinPrep® preparations fixed with 95% alcohol could be evaluated for ER and PgR statuses using both liquid suspension and dry slides over 56 days at RT.[14] Moreover, Hansen et al. revealed that CytoRich-Red-fixed SurePath™ samples could be stored in a refrigerator on dry slides for at least 40 days without loss of staining intensity for many primary antibodies.[19] Further investigation of fixatives and storage temperatures is essential.

Tissue blocks preserve the antigenicity of samples for several decades under normal storage conditions, whereas in tissue sections, it can be affected by oxidation, drying, exposure to fluorescent or sunlight, and environmental temperature and show decreased immunoreactivity over time.[20,21] We speculated that, similar to tissue sections, storage on slides was more susceptible to oxidation and various environmental effects than storage in a cell preservation medium, resulting in antigen degradation. Therefore, to preserve the immunoreactivity for samples, we recommend storage in CP vials until just before performing ICC.

In this study, we used primary antibodies that are frequently used in our laboratory and have different antigenic localization (the nucleus, cell membrane, and cytoplasm). In the future, it will be necessary to expand the scope of examination, such to determine cancers of unknown primary origin, differentiate between mesothelioma and adenocarcinoma, and differentiate between lymphoma and sarcoma.

Conclusion

Samples stored in CP vials at RT revealed more stable immunoreactivity than those on CP slides under refrigeration. The ICC controls for TTF-1, CEA, and the p16/Ki67 cocktail markers had stable immunoreactivity for at least 6 months when stored in CP vials at RT and 3 months for GATA-3. The findings of this study are useful for the collection of clinical samples to be used as controls. Further investigation for additional markers is indispensable for the clinical application of ICC for LBC using CP.

AUTHOR CONTRIBUTIONS

Ayumi Ryu: Conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing - original draft, and writing - review and editing. Keiichiro Honma:

Conceptualization, formal analysis, methodology, project administration, writing - review and editing. Yoshimi Umeno: Investigation and validation. Fusayo Uefuji: Investigation and validation. Takako Muramatsu: Investigation and validation. Sanako Nishimura: Methodology and validation. Satoshi Tanada: Project administration. Takashi Yamamoto: Project administration. Shigenori Nagata: methodology, writing - review and editing. Tomoyuki Yamasaki: Project administration. Masayuki Ohue: Conceptualization, methodology, project administration, supervision, writing - review and editing.

ACKNOWLEDGEMENTS

We would like to thank Editage (www.editage.com) for their assistance with English language editing.

FUNDING INFORMATION

No funding was received for this study.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure Legends

Figure 1 Workflow for evaluating suitable sample storage conditions for ICC controls

Figure 2 The ICC for TTF-1 under various storage conditions, using scraping of a lung adenocarcinoma lesion.

Immunoreactivity in the CP vials stored at RT showed that the first ICC(A), second ICC(B) (RT for 103 days) and third ICC (C) (RT for 256 days) was maintained. (D) A second ICC for a CP slide stored under refrigeration showed decreased positivity as compared to the first ICC. (E) Further reduction in immunoreactivity was noted in the third ICC for the CP slide stored under refrigeration for 256 days.

(A–E ICC, counterstained with hematoxylin, ×200). ICC, immunocytochemistry; CP, Cellprep®; RT, room temperature.

Figure 3 p16/Ki67 cocktail dual staining under various storage conditions using cervical adenocarcinoma cells.

(A) In tumor cells, the nucleus and cytoplasm (brown) were stained using p16 and the nucleus (red) was stained using Ki67, within the same cell, and dual staining was determined as positive. (B) In the second ICC for the CP vial stored at RT for 146 days, dual staining was positive. (C) In the third ICC for the CP vial stored at RT for 256 days, it was positive, similar to the first ICC. (D) In the second ICC for the CP slide stored in the refrigerator for 146 days, it was positive, similar to the first ICC. (E) In the third ICC for the CP slide stored under refrigeration for 256 days, dual staining was weakly positive. Stainability by p16 was maintained, whereas the red color of the nucleus by Ki67 was attenuated. (A–E dual staining, counterstained with hematoxylin, ×400). ICC, immunocytochemistry; CP, Cellprep®; RT, room temperature.