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Original Article

DNA flow cytometry analysis in body cavity fluids using liquid-based cytology

Short Title: DNA flow cytometry using liquid-based cytology

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Abstract

Background: DNA flow cytometry using LC-1000 is utilized for analyzing cellular and nuclear lengths and DNA content in individual cells with samples in cell preservation solutions to acquire the cell proliferation index (CPIx). This study aimed to evaluate the performance of body cavity fluid cytology using LC-1000. As cell preservation solutions, liquid-based cytology (LBC) by Cellprep® (CP) was compared with CelVerse™ (CeV), specifically designed for the LC-1000.

Methods: Overall, we evaluated 43 samples (20 malignant and 23 benign) of body cavity fluids suspended in CeV and 59 samples (28 malignant and 31 benign) suspended in CP. The CPIx was compared between malignant and benign cases of CeV and CP. Further, the subject groups were divided according to the SubG1% in a sample.

Results: The CPIx in CeV was 0.14–10.22 (median 0.62) in malignant cases and 0.09–4.64 (median 0.19) in benign cases ($p < 0.001$); additionally, CP displayed 0.14–9.87 (median 0.84) in malignant cases and 0.11–3.02 (median 0.22) in benign cases ($p < 0.001$). The area under the curve (AUC) was 0.811 for CeV and 0.776 for CP. The difference in SubG1% between the CeV and CP groups was statistically significant ($p < 0.0001$), and influenced the AUC in CP.

Conclusion: The CPIx in CeV or CP samples using the LC-1000 may be applicable as an objective indicator in the body cavity fluid cytology. Samples preserved in CeV provided more consistent results, unaffected by SubG1%, when compared to those stored in CP.

Keywords: DNA, flow cytometry, liquid-based cytology, body cavity fluids

Total Number of Each

- 1) Text pages, including title page, abstract, main text, references, tables and figure legends: 16 pages
- 2) Tables: 6
- 3) Figures: 5

Introduction

Flow cytometry (FCM) has been used to detect hematological and non-hematological neoplasms. Non-hematologic neoplasms are detected using routine FCM analysis in clinical practice,^{1,2} FCM analysis using proprietary antibody panels,^{3,4} or the identification of aneuploid cell populations with DNA FCM analysis.⁵⁻⁷

LC-1000 (Sysmex Corp., Kobe, Japan) is an FCM-based device that analyzes cellular and nuclear lengths and DNA content in individual cells and acquires the cell proliferation index (CPIx), which is a unique index. The LC-1000 analyzes samples placed in an alcohol-based cell preservation solution. Therefore, the LC-1000 has the potential to perform both cytological examination and FCM analysis in the same liquid-based cytology (LBC) sample. Its applicability has already been reported for cervical cancer screening.⁸⁻¹⁰ In recent years, there have also been reports on non-gynecological materials using LC-1000.¹¹⁻¹³ The reports related to LC-1000 have been analyzed using ThinPrep vials (Hologic).⁸⁻¹⁰

The cytological diagnosis of body cavity fluids, including pleural and peritoneal lavage, has a significant impact on the clinical stage, clinical management, and prognosis.¹⁴⁻¹⁶ Therefore, body cavity fluid cytology must be more objective and reproducible. Recently, the International System for Reporting Serous Cytology (TIS) has been proposed as a standard reporting format, and its usefulness has been reported. However, it was noted that there was higher heterogeneity in the “inadequate” and “atypical” categories when compared to the “benign,” “suspicious,” and “malignant” categories.¹⁷ Poor interobserver agreements were reported for the “atypical” and “suspicious” categories in TIS.¹⁸

We investigated whether exfoliative cell analysis using LC-1000 with LBC samples could be applied to body cavity fluid cytology to detect tumor cells, aiming for an objective indicator of cytological diagnosis. Two cell-preservation solutions were used in this study. One is CelVerse™ (CeV; Sysmex Corp., Kobe, Japan),¹⁹ which is the cell preservation solution dedicated for the LC-1000, and the other is the Cellprep® (CP; Roche Diagnostics, Tokyo, Japan) vial for the CP LBC system. The analytic performance of the LC-1000 in CP samples was evaluated and compared to that of CeV.

Materials and Methods

Sample preparation

Body fluid samples (pleural effusion, ascites, and pleural or peritoneal lavage fluid) were provided to the cytological laboratory for cytological diagnosis and centrifuged at $850 \times g$ for 3 min to prepare a specimen. After cytological diagnosis, the residual materials were divided into cell preservation solutions for analysis by LC-1000 and vials for LBC using CP. Two types of solutions were used for LC-1000 analysis. One was CeV, which is a methanol-based cell preservation solution, and the other was Cellprep® LBC Solution—Body Fluid, FNA for other samples (CP vial; Roche Diagnostics, Tokyo, Japan), which is an ethanol-based cell preservation solution. Samples for LC-1000 analysis were suspended in CeV or CP and stored in a refrigerator. LC-1000 analysis of CeV was performed within 1–8 days (mean 4.0, median 4 days). CP was performed within 2–13 days (mean 5.7, median 5 days). Papanicolaou (Pap) slides were prepared with LBC vials using CP to confirm the presence of tumor cells in malignant samples and the absence of tumor cells in benign samples in the residual material. Immunocytochemistry (ICC) analysis was conducted on some samples using antibodies against Claudin 4, Thyroid transcription factor-1 (TTF-1), p40, Insulinoma-associated protein 1 (INSM1), PAX8, GATA binding protein 3 (GATA3) and Carcinoembryonic antigen (CEA) (Figure 1). As this study aimed to investigate the adaptability of CP samples to body-cavity fluids using the LC-1000, only the samples with clearly malignant or benign results on body fluid cytology were analyzed. Specimens with equivocal cytology results were excluded. The cytological results obtained from the clinical cases were used as standards. Of the 59 samples suspended in CeV, 43 had sufficient cell counts for LC-1000 analysis, and the CPIx was calculated. The 43 CeV samples comprised 20 malignant and 23 benign samples. Of the 70 samples suspended in the CP vials, 59 had sufficient cell counts. The 59 samples in the CP vials comprised 28 malignant and 31 benign samples. Pooled samples were prepared to ensure sufficient cell numbers for the analysis (Table 1). The number of samples in CP was larger than that in CeV as the main purpose of this study was to examine whether the LBC (CP) used in our institute could be adapted to the LC-1000. Fifteen samples (eight malignant and seven benign) were analyzed using cell preservation solutions from both the CeV and CP vials. Eight malignant samples were adenocarcinomas (5 stomach, 2 lung, 1 bile duct). One of the five adenocarcinomas originating from the stomach was prepared by mixing with a benign sample.

To examine the effect of the number of malignant cells in malignant samples, we mixed malignant CP samples with benign CP samples to create samples with an artificially reduced number of cells and verified their effect. The number of malignant cells per a CP specimen was quantified as 0 (no malignant cells), 1+ (20-200 cells), or 2+ (>200 cells), and the CPIx values were compared. Samples containing fewer than 20 cells were

excluded due to uncertainty. Of the total 66 samples, 36 samples were 2+, 8 samples were 1+, and 22 samples were 0.

LC-1000 analysis

The samples were mixed by inverting the tubes, and 5 mL of the solution was collected and placed in a test tube. The test tubes were centrifuged at 420 $\times g$ for 5 min. The supernatant in the test tube was removed, and the sample volume was adjusted to 2.5–3 mL and stirred with a dropper. The pretreated samples were placed on an LC-1000 column.

The steps described below were conducted using LC-1000. The cells in the sample solution were mechanically and ultrasonically dispersed to reduce cell aggregation without compromising cell morphology. The sample solution was passed through a metal filter with a pore size of 10 μm to remove blood cells and debris < 10 μm . RNase solution (final concentration 5 units) was added to the sample solution discharged after pretreatment and incubated at 37 °C for 10 min. DNA staining was performed by adding propidium iodide to a final concentration of 20 $\mu\text{g/mL}$ and incubating for 1 min. The LC-1000 detected forward and side-scattered fluorescence signals produced by 30 mW laser illumination at 488 nm using FCM and provided information on individual cells. Consequently, three cellular parameters (DNA content, cellular and nuclear lengths) were measured, and the nuclear/cytoplasmic (N/C) ratio was calculated from the area of the nucleus and cell. The measurement time, including pretreatment, was approximately 40 min. Among 23 items obtained from the LC-1000, the following items defined in the manufacturer's instructions were used in this study: CPlx, the number of cells with higher DNA content than that in the G0/G1 phase compared with the number of cells corresponding to the G0/G1 phase (diploid) within the cell population with a high N/C ratio. SubG1%, percentage of the number of the SubG1 phase corresponding to the number of single epithelial cells (EC). The signal area with a lower fluorescence intensity than that of the G1 phase peak was classified as the SubG1 phase. The CPlx is a unique indicator for evaluating the proliferative status of tumor cells.¹⁰⁻¹². In addition, to eliminate the influence of tetraploid cells, which are also present in normal cells, we calculated the number of aneuploids (including polyploids) by subtracting diploids, tetraploids, and SubG1 from EC. Then, we calculated the area under the curve (AUC) of Cev and CP for the ratio of aneuploids to EC (Aneuploid%).

Statistical analysis

The CPlx or Aneuploid% for malignant and benign samples suspended in CeV or CP was analyzed using the Mann–Whitney U test. The receiver operating characteristic (ROC) curve of CPlx or Aneuploid% was created based on the cytological results. The CPlx or Aneuploid% with the highest sensitivity and specificity in the ROC curve was set as the cut-off value. The AUC is an evaluation indicator for binary classification models, and the closer this value is to 1, the higher the model performance. The AUC was used to evaluate the predictive ability of CPlx and Aneuploid% for the tumor cell detection in body cavity fluids followed by the comparison of the AUCs between CPlx and Aneuploid%.

The correlation of CPlxs in 15 paired samples from CeV and CP vials was analyzed using Spearman's rank correlation coefficient.

SubG1% was compared in malignant and benign samples in CeV and CP vials and analyzed using the Mann–Whitney U test. In addition, the SubG1% was compared between 2 and 7 days and 8 and 13 days of storage periods in CP. To investigate the influence of cells classified SubG1 phase, AUCs calculated from the CPlx values for CP and CeV were evaluated according to the percentage of SubG1 phase cells in a sample. The influence of SubG1% for AUCs was analyzed using Spearman's rank correlation coefficient.

The comparison of CPlxs based on malignant cell count was performed using the Mann–Whitney U test.

The p values <0.05 (two-sided) in the Mann-Whitney U test were considered statistically significant.

All statistical analyses were performed using Easy R (Saitama Medical Center, Jichi Medical University), which is a graphical user interface for R.²⁰

Results

The comparison of CPlx or Aneuploid% for samples stored in CeV or CP is presented in Table 2. The CPlx or Aneuploid% for malignant and benign samples stored in CeV or CP were statistically significant ($p < 0.001$).

Regarding CPlx values, AUCs for CeV and CP were 0.811 and 0.776, respectively. When the cutoff value for samples stored in CeV was 0.42, the sensitivity and specificity were 70.0 and 87.0%, respectively. However, in CeV, there were three benign samples with a CPlx ≥ 0.42 and six malignant samples with a CPlx < 0.42 . When the cutoff value for CP was 0.34, the sensitivity and specificity were 78.6 and 67.7%, respectively. However, in CP, there were 10 benign samples with a CPlx ≥ 0.34 and 6 malignant samples with a CPlx < 0.34 . The AUC between CPlx and Aneuploid% was not statistically significant for either CeV ($p = 0.244$) or CP ($p = 0.625$) (Table 2; Figure 2).

The correlation coefficient between CeV and CP for the 15 samples was 0.907 ($p < 0.0001$).

The CPIxs values for each primary lesion and histological type in the malignant cases are presented in Table 3. For both CeV and CP, the CPIxs of cases with primary bile duct lesions (cholangiocarcinoma) were lower than the median of those in benign samples. Variations in the CPIxs were observed even in the same primary lesions.

SubG1% was compared between CeV and CP, and a statistically significant difference was observed ($p < 0.0001$) (Figure 3). The variation in SubG1% was more pronounced in CP than in CeV (Table 4). The outlier was defined as values greater than the “third quartile + interquartile range (IQR) $\times 1.5$.” The outlier of SubG1% for CP was ≥ 73.0 . The corrected AUC for CP was calculated by excluding samples ($n = 5$) with SubG1% outliers. The corrected AUC for CP was 0.837 (95% confidence interval, 0.728–0.946), with a cutoff value of 0.340, sensitivity of 77.8%, and specificity of 77.8%. The SubG1% showed no statistically significant differences between malignant and benign samples for either CeV ($p = 0.237$) or CP ($p = 0.058$) (Table 4), and between 2 and 7 days ($n = 42$) and 8 and 13 days ($n = 17$) of storage in CP ($p = 0.103$). The AUC was calculated when the samples were limited by SubG1%, and the relationship between the degree of SubG1% and AUC was expressed as a correlation coefficient. Additionally, the effect of restricting samples by SubG1% on the AUC was expressed as the coefficient of variation (CV). The CVs for the AUC were 1.63% for CeV and 3.71% for CP (Table 5).

The comparison of CPIxs according to the number of malignant cells was presented in Figure 4. In the semi-quantification of cell counts, there was a statistically significant difference between 2+ and 1+ vs. 0 ($p < 0.05$), 2+ vs. 0 ($p < 0.01$), 2+ vs. 1+ ($p < 0.05$), but not between 1+ vs. 0 ($p = 0.80$).

Discussion

The evaluation of body cavity fluid cytology, including pleural and peritoneal lavage, has a significant impact on patient prognosis, treatment plans, and staging. The aim of this study was to expand the range of applications of LBC by using it for DNA FCM. The analysis of DNA content in body cavity fluids by FCM have been reported previously; however, fresh samples were used (Table 6).^{5-7, 21-30} This may be a problem in some laboratories outside working hours (after normal working hours and on weekends and holidays) and if the samples need to be sent to a reference laboratory.⁷ Once samples have been placed in cell preservation solutions, such as CeV or CP, they do not need to be processed immediately. There are several reports of DNA FCM using LC-1000 for gynecological LBC materials,⁸⁻¹⁰ but very few reports on body cavity fluids.¹¹ In this study, we did not target a specific primary malignancy, but instead subjected tumor cells from any primary malignancies that could

potentially appear in body cavity fluids to be able to respond to any situation. There are few reports of DNA flow cytometry using LBC samples with body cavity fluids, and thus far, no fundamental studies have been performed. This study subjected clearly malignant and benign samples using the ICC when necessary, and did not include equivocal samples.

The CPlx was significantly different between the malignant and benign samples stored in CeV or CP ($p < 0.001$), with AUC values of 0.811 and 0.776 for CeV and CP, respectively (Table 2). In addition, the CPlx values of CeV and CP exhibited excellent correlations. According to various studies, most reports have shown that the specificity was excellent,^{21,22,26,29} but the sensitivity was inferior to that of cytological morphology^{5,21,27,29} or image cytometry.^{7,26} However, combined cytology and ploidy had higher sensitivity than cytology alone, demonstrating the usefulness of DNA-FCM as an adjunct to cytology for diagnosing malignancy in effusions.^{25,27} In this study, the CPlx that yielded the highest sensitivity and specificity based on the ROC curve was set as the cutoff value. However, the use of the CPlx in screening body-cavity fluid cytology requires further investigation. Our results are based on a limited sample size and need to be verified against a larger sample size.

In both CeV and CP samples, there were benign samples with high CPlx values and malignant samples with low CPlx values. This can be attributed to several factors. The CPlx is defined as the number of cells with a higher DNA content than that in the G0/G1 phase when compared with the number of cells corresponding to the G0/G1 phase (diploid) within the cell population with a high N/C ratio. Cells with higher DNA content than that in the G0/G1 phase can include cells in the S, G2/M (tetraploid), polyploid, and aneuploid phases. In the DNA diploid G2/tetraploid G1 region of a DNA histogram, G1/G0 tetraploid cells and G2/M diploid cells coexist.³¹ Biesterfeld et al. investigated the prevalence and content of ploidy in various specimens, including cytopsin preparations from pleural and peritoneal fluids.³² They reported that on 46 Feulgen stained non-neoplastic cytopsin preparations from pleural or peritoneal fluids, polyploidy was observed in three cases (5–6%), with the highest ploidy level being 8–75 c. Lazcano et al. reported that an important finding in DNA image analysis was the presence of tetraploidy in benign fluid. Reactive mesothelial cells can have several nuclei and even exhibit mitotic figures.⁷ They noted that the difference between benign tetraploid mesothelial cells and malignant tetraploid cancer cells is the lower proportion of S-phase fraction in the benign cases.⁷ Normal human diploid cells subjected to senescence-inducing stimuli undergo a temporary cell cycle arrest in the G2 phase and then progress to the G1 phase without cell division, escaping mitotic phase results in tetraploid G1 cells, causing irreversible cell growth arrest.³³ Activated mesothelial or histiocyte cells or the presence of senescent cells may

increase the CPlx even in benign samples.

We excluded the effect of tetraploid cells, calculated the Aneuploid%, and compared the AUC for Aneuploid% with that for the CPlx. There was no significant difference between the AUC for CPlx and that for Aneuploid%.

In a control population with ovarian cysts or enlarged uteri, aneuploid cell populations were present in 7/20 specimens.²³ Sayed et al. reported that 29.7% (11/37) of the benign group samples were aneuploid.²⁸ Krishan et al. showed that an aneuploid was recognized in 17.7% (11/62) of benign specimens and 38.2% (13/34) of malignant specimens.²⁷ The results of the present study may be attributed to the fact that some degree of aneuploidy is observed even in benign cases.

Malignant samples, such as cholangiocarcinoma with low CPlx values, were present in both CeV and CP. Some malignant tumors have been reported to be diploid; 30–36% of malignant cases have been interpreted as diploid.^{7,29,34} Kubota et al. investigated the effect of chemotherapy on DNA ploidy in gastric and colorectal carcinomas.³⁵ They concluded that the response of gastric and colon cancer cells to preoperative chemotherapy could be determined by observing the change in the DNA ploidy pattern from aneuploid to diploid. In this study, malignant cases with low CPlx values may have been diploid tumors or in a state where the tumor cells had changed from aneuploid to diploid owing to chemotherapy.

The difference in CPlxs was compared based on the number of malignant cells; the results indicated that the CPlx was unsuitable for detecting small numbers (1+) of malignant cells. In DNA FCM, the presence of a few malignant cells among a large number of benign mesothelial cells can make it difficult to obtain a significant aneuploidy peak.³⁶ Because the analysis on the LC1000 can be completed in a short time (approximately 40 min including pretreatment), the LC1000 results can be referred to when performing a Pap test. In particular, we believe that the LC1000 may be an indicator when target cells are present in some numbers but difficult to identify.

We noted a statistically significant difference in the percentage of cells classified in the SubG1 phase between the samples stored in CeV and CP. The SubG1 phase is considered as an indicator of apoptosis and contains fragmented DNA that shows weak fluorescence staining intensity. We speculated that the extremely high SubG1% in the samples might be due to the influence of the cell preservation solution. CeV is a methanol-based cell preservation solution and CP is an ethanol-based cell preservation solution. Previous studies with the LC-1000 have used LBC samples by Thinprep®.⁸⁻¹⁰ The PreservCyt® solution used in Thinprep® is a methanol-

based cell preservation solution. This is the first study to examine the diagnostic performance of the LC-1000 for CP. Considering the possibility that the CP solution may cause instability in fluorescence staining, the AUC of the CPlx value was analyzed by dividing the target samples into 10% increments of SubG1%. A negative correlation was observed between SubG1% and AUC for both CeV and CP. The coefficient of variation for the AUC was 1.56% for CeV and 3.71% for CP (Table 5). When using CP in the LC-1000 analysis, the interpretation of CPlx needs to be considered, taking into account SubG1%. In this study, we recommend removing samples with at least outliers of SubG1% $\geq$ 73.0. Regardless of the use of CeV or CP, the DNA histogram in addition to the measurement data should be checked to confirm the cell cycle status (Figure 5).

Conclusion

The CPlx of body cavity fluid cytology using CeV or CP with the LC-1000 may be applicable as an objective index when evaluated based on DNA histograms. Although the CPlx values of CeV and CP highly correlated, more stable results were obtained for the samples stored in CeV than for those stored in CP. This indicates an expansion of the applicability of LBC.

Data availability statement

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

Funding statement

The authors did not receive any funding.

Conflict of interest disclosure

The authors have no conflicts of interest to declare.

Disclosure

This study was conducted based on a collaborative research agreement between Osaka International Cancer Institute and Sysmex Corporation. The study was designed at Osaka International Cancer Institute. Blinded samples were measured at Sysmex Corporation. Services and materials were provided by Sysmex Corporation.

Ethics approval statement

This study was approved by the Ethics Committee of Osaka International Cancer Institute (Approval #19134, #20249).

Author Contributions

Ayumi Ryu: Conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing – original draft preparation, and review and editing. **Keiichiro Honma:** Conceptualization, methodology, project administration, writing, and review and editing. **Sayoko Tsuzaki:** Investigation and validation. **Risa Yoshioka:** Investigation. **Satoshi Tanada:** Project administration. **Takashi Yamamoto:** Project administration. **Hidetoshi Satomi:** Writing and review and editing. **Shigenori Nagata:** Writing and review and editing. **Tomoyuki Yamasaki:** Project administration. **Masayuki Ohue:** Conceptualization, methodology, project administration, supervision, writing, and review and editing.

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Figure Legends

Figure 1. Sample processing workflow for dividing residual materials for the diagnostic and LC-1000 samples.

Figure 2. Cell morphology of an ascites sample and the DNA histogram of cells with high N/C ratio. This is a case of poorly differentiated stomach adenocarcinoma.

A: Cells with eccentric nuclei are small and scattered, and must be distinguished from histiocytes (conventional specimen, Papanicolaou test, original magnification $\times 400$).

B: Cells with eccentric nuclei had foamy cytoplasm, prominent nucleoli, and irregular nuclei (liquid-based specimen (Cellprep®), Papanicolaou test, original magnification $\times 1000$).

C: The distribution of cell length and N/C ratio (left) and DNA histogram of cells with high N/C ratio (right) in a Cellprep® sample were presented. The number of cells equivalent to the G0/G1 phase (blue) was 380, and the number of cells with higher DNA content than that in the G0/G1 phase (red) was 517. The CPlx was 1.36. N/C, nuclear/cytoplasmic ratio; CPlx, cell proliferation index.

Figure 3. Comparison of SubG1% between CelVerse™ (n = 43) and Cellprep® (n = 59) samples. The SubG1% was more stable in CelVerse™ than in Cellprep®. A statistically significant difference was observed between CelVerse™ and Cellprep® (p < 0.0001). CeV, CelVerse™ ; CP, Cellprep®.

Figure 4. Comparison of semi-quantified malignant cell counts and CPlxs, using the Cellprep® solution

Number of malignant cells per a Cellprep® specimen was quantified as 0 (no malignant cells), 1+ (20-200 cells), or 2+ (>200 cells).

CPlx, cell proliferation index; ✕, statistically significant.

Figure 5. DNA histograms of CelVerse™ and Cellprep® in the same benign case

A: Many mesothelial cells are observed. No malignant cells were detected. (Liquid-based specimen (Cellprep®), Papanicolaou test, original magnification $\times 600$).

B: The distribution of cell length and N/C ratio (left) and the DNA histogram of cells with high N/C ratio (right) in a CelVerse™ sample were presented. The number of cells equivalent to the G0/G1 phase (blue) was 3018, and the number of cells with a higher DNA content than the G0/G1 phase (red) was 605. The CPlx was 0.20. The

number of cells corresponding to the SubG1 phase (yellow-green) was 86 (1.9% of single epithelial cells).

C: The distribution of cell length and N/C ratio (left) and the DNA histogram of cells with high N/C ratio (right) in a Cellprep® sample were presented. The number of cells equivalent to the G0/G1 phase (blue) was 241, and the number of cells with higher DNA content than that in the G0/G1 phase (red) was 94. The CPlx was 0.39. The number of cells corresponding to the SubG1 phase (yellow green) was 195 (18.2% of single epithelial cells), which was higher in Cellprep® than in CelVerse™, although these were in the same case. The CPlx was higher in a Cellprep® sample than in a CelVerse™ sample. N/C, nuclear/cytoplasmic ratio; CPlx, cell proliferation index.