Direct Next-generation Sequencing Analysis using Endometrial Liquid-based Cytology Specimens for Rapid Cancer Genomic Profiling

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Short running title: Rapid NGS in endometrial LBC specimens

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Abstract

Background: Genomic examination of cytology specimens is often performed on cell blocks or conventional smears rather than on liquid-based cytology (LBC) specimens. Since LBC specimens preserve high-quality DNA, cancer genome profiling using next-generation sequencing (NGS) is also attainable from residual LBC specimens. One of the advantages of using LBC specimens for NGS is that it allows direct extraction of DNA from residual specimens, avoiding a sacrifice of smear slides and minimizing genomic profiling processing time.

Methods: Endometrial LBC specimens were subjected to NGS analysis to validate the practicality of rapid cancer genomic profiling in a pathology laboratory. The extracted DNA was subjected to NGS using a customized cancer gene panel comprising 56 genes and 17 microsatellite regions. The workflow strategy was defined, and the processing time estimated for specimen sampling, cell counting, NGS run, and genome profiling.

Results: NGS analysis of most LBC specimens revealed somatic mutations, tumor mutation burden, and microsatellite instability, which were almost identical to those obtained from formalin-fixed paraffin-embedded tissues. The processing time for direct NGS analysis and cancer genomic profiling of the residual LBC specimens was approximately five days.

Conclusion: The residual LBC specimens collected using endometrial cytology were verified to carry a high tumor fraction for NGS analysis and could serve as an alternate source for rapid molecular classification and diagnosis of endometrial cancers, as a routine process in a pathology laboratory.

Key words: Next-generation sequencing, Endometrial cancer, Liquid-based cytology, Cancer panel, Rapid genomic profiling

Introduction

Cytology is less invasive and expensive compared to histological examination. Cytology specimens are beneficial for integrative cancer diagnosis in medical laboratories since they can be used for immunocytochemical, biochemical, and molecular analyses.[1-3] Liquid-based cytology (LBC) has recently been used for immunocytochemical studies, as well as for routine cytological diagnosis including endometrial cytology.[4-6] Since LBC specimens can preserve high DNA quality even after 5 years of storage,[7] LBC specimens can be used in cancer molecular analysis via next-generation sequencing (NGS).[8-10] Normally, conventional cytology smears and cell block (CB) preparation are more routinely used for NGS analysis with some advantages.[11-15] However, residual LBC specimens, when available, could also be exploited to minimize the use of smear slides since they can be directly and rapidly used for DNA extraction without any additional procedures such as cell scraping and CB preparation.[13, 14]

A previous study demonstrated that human cultured cells prepared for LBC and clinical LBC specimens were suitable for direct DNA extraction in cancer genomic panel analysis using NGS.[7] Furthermore, the feasibility of LBC specimens for NGS analysis was validated by retrospective studies using residual LBC specimens obtained

from various organs, such as the thyroid gland, breast, endometrium, and lymph nodes after long-term storage.[16, 17] The present study sought to expand the above methods by using a modified custom cancer gene panel[16] to verify whether direct NGS from residual LBC specimens could be practically applied as a standard, routine technique in laboratories.

Materials and Methods

Clinical specimens

Clinical specimens registered in the Clinical Research of Cancer Gene Panel Analysis Study from January 2019 to January 2021 at Kagoshima University Hospital were used in this study. The study samples included 23 endometrial LBC specimens and 43 corresponding FFPE tissues obtained by biopsy and/or by surgical resection (66 samples in total). The tissues were fixed with phosphate-buffered neutral 10% formalin for 24 h, processed for paraffin embedding, and sectioned for hematoxylin and eosin staining. The endometrial cytology specimens were obtained using a specific device (Endocyte® sampler, Laboratoire CCD, Paris, France).[18, 19] The cells were immediately fixed with CytoRich Red solution (Becton Dickinson, Franklin Lakes, NJ, USA), processed using a BD SurePath liquid-based Pap Test System (Becton Dickinson), and

stained with Papanicolaou staining solution. Residual LBC specimens were stored at 4°C until further analysis to reduce DNA degradation.

DNA extraction and quality check

DNA from FFPE tissues was obtained from 3–6 sections with of 10 μm thickness, including more than 30% of the cancer area. DNA extraction from FFPE and LBC specimens, as well as DNA quality monitoring were performed as previously reported.[7, 16]

Customized gene panel design

A cancer panel was redesigned with a minor modification to the previous panel[4] by including 56 cancer-related genes and 17 microsatellite foci that were selected from the QIAseq Targeted DNA Custom Panel (Qiagen, Reston, VA, USA), 2,640 primers for the regions of interest (194,131 bp), and an average exon coverage of 99.87%. This panel includes genes for the World Health Organization (WHO) molecular classification for endometrial cancer (Supplemental Table S1). The WHO classification is composed of four molecular types for endometrioid carcinoma: *POLE* mutation (*POLE*mut), MMR-deficient (MMR-d), *TP53* mutation (p53mut), and no specific molecular profile (NSMP).[20]

NGS analysis

NGS library generation and quality checks were performed as previously described.[7, 16] The libraries were applied to a MiSeq sequencer (Illumina, San Diego, CA, USA) after dilution with a hybridization buffer to a final concentration of 20 pM. Sequencing data were analyzed using the Qiagen web portal service (https://www.qiagen.com./us/shop/genes-and-pathways/data-analysis-center-overview-page/), which indicated the necessity of a mean sequence depth of 5,000 for detection of 1% variant allele frequency (VAF). The cut-off value of VAF was inversely proportional to the mean sequence depth. Since the NGS condition yielded a mean depth of 500, a VAF greater than or equal to 10% was considered to be significant. The human genome reference GRCh37 hg19 (https://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.13/) and the Catalogue of Somatic Mutations in Cancer (COSMIC ver. 90, https://cancer.sanger.ac.uk/cosmic) databases were used as the analytical references.

Calculation of TMB and MSI scores

The number of missense mutations, including non-synonymous mutations and internal deletions, were counted as somatic mutations, and tumor mutation burden (TMB) was calculated as the number of single nucleotide variants/Mbp of DNA sequence.[21, 22] Microsatellite instability (MSI) scores were determined using MSIsensor (ver. 1.0).[23, 24] To determine the cut-off values of MSI and TMB using receiver operator

characteristic curves, 59 endometrial cancer FFPE samples were used. The TMB values of wild- and mutation-type *POLE* cases were 3.63 ± 3.8 (mean ± standard error) and 175.1 ± 61.0 ($p = 0.026$, Welch's *t*-test), respectively, and the cut-off value for TMB-ultrahigh (TMB-UH) was estimated as 72. Among the wild-type *POLE* cases, the TMB values in MMR-proficient (MMR-p) and MMR-deficient (MMR-d) cases were 22.5 ± 3.0 and 63.0 ± 5.1, respectively ($p < 0.001$, Welch's *t*-test), and the cut-off values for TMB-high (TMB-H) and -low (TMB-L) were estimated to be 42. Similarly, in wild-type *POLE* cases, the MSI values in MMR-p and MMR-d cases were 3.6 ± 0.5, and 38.7 ± 2.0, respectively ($p < 0.001$, Welch's *t*-test), and the cut-off values for the differentiation of MSI-high (MSI-H) and MSI-low (MSI-L) were estimated to be 13.

Pathological diagnosis and cell counting in LBC specimens

Pathological diagnosis was carried out by a group of board-certified cytotechnologists (TA and YNK), cytopathologist (IK), and pathologists (IK and HT). The cell numbers were counted by a skillful cytotechnologist (TA) using SurePath Papalicolau stained slides newly prepared from the residual LBC samples. Tumor cell number (T) was counted under five high-power fields (HPFs) at 400× magnification. The non-tumor cell count (N) was calculated from an average of five HPFs, from which the tumor cell to non-tumor cell ratio and tumor fraction (T/T+N) were calculated.

Estimated tumor cell numbers subjected to NGS were calculated as following formula: Average cell count × [SurePath spot area (113.04 mm^2) / 400× HPF area (0.2376 mm^2)] × [LBC sample volume used for NGS (1 mL) / LBC sample volume used for SurePath preparation (0.25mL)] × [applied volume of PCR library for NGS (μL) / fixed total volume for NGS reaction (16.8 μL): these volumes are indicated by QIASeq DNA panel system[7, 16]]. For example, the estimated tumor cell number of case #1 (Table S2) was calculated as 43,429 = 127.8 × [113.04/0.2376] × [1 mL/0.25 mL] × [3 μL /16.8 μL], and that of case #18 (Table S2) was 62,419 = 134.4 × [113.04/0.2376] × [1 mL/0.25 mL] × [4.1 μL /16.8 μL].

Immunohistochemistry with FFPE section

FFPE tissue sections were subjected to immunohistochemistry (IHC) using antibodies against MLH1 (clone ES05 M3640), MSH2 (clone FE11 M3639), MSH6 (clone EP49 M3646), and PMS2 (clone EP51 M3647) purchased from DAKO (Tokyo, Japan). Staining was performed using the Envision FLEX High pH K8000 system (DAKO). Positive nuclear staining of lymphocytes in tissue sections was used as a positive control. MMR-d was defined as complete loss of nuclear staining for both MLH1 and PMS2, both MSH2 and MSH6, MSH6 only, or PMS2 only.

Ethics approval

The study was approved by the Ethics Committees for Clinical and Epidemiologic Research at Kagoshima University Hospital (approval number: #180215, modified), and written informed consent was obtained from each participant.

Results

Workflow of NGS analysis using LBC specimens

NGS analysis using LBC specimens was completed within 4 days, starting from DNA extraction, quality check, preparation of NGS library and quality check, NGS run, and annotation of gene alterations. Since cytological diagnosis of LBC (processed by BD SurePath liquid-based Pap Test System) will take one day after endometrial sampling at the outpatient clinic, the overall turnaround time (TAT) from sampling to genome profiling was estimated to be 5 days. The workflow is illustrated as shown in Figure 1.

Tumor cell number, tumor fraction, and DNA quality of LBC specimens for NGS

The tumor cell number, tumor fraction, DNA quality (QC score and DNA yield of NGS library), and input DNA subjected to NGS are summarized in Table S2. Among 23 cases, only one case (no. 21) failed to detect any mutations, MSI, and TMB, even though the sample contained 9,644 tumor cells with 15% tumor fraction. Three cases

(nos. 3, 10, 14) failed to detect either mutation or MSI, which contained 6,326, 39,583, and 30,818 tumor cells equivalent to 13%, 24%, and 26% tumor fractions, respectively. From the other 19 cases, successful NGS results were obtained, which were almost similar to those from FFPE. In these 19 LBC specimens, the tumor cell number ranged from 9,900 to 245,000, and the tumor fraction ranged from 50% to 98%, except for one case (no. 6) that contained 5,267 tumor cells with only 2.7% tumor fraction. The distribution of case numbers and frequency of successful NGS was stratified according to tumor fraction and tumor cell numbers (Table 1), showing that input specimens with more than 10,000 tumor cells and more than 50% tumor fraction resulted in a 100 % success rate for NGS. No samples showed a QC score greater than 0.04, and insufficient DNA recovery lower than 1 ng/μL in either LBC or FFPE specimens. The input DNA was much lower in LBC-based NGS (11–64 ng) than that in FFPE-based NGS (25–168 ng). Representative microphotographs of the LBC specimens of both failed and successful NGS are shown in Figure 2.

Genomic profiles of endometrial cancer

The genomic profiles evaluated by NGS from LBC and the corresponding FFPE specimens along with pathological diagnosis are summarized in Table S2. Among the 23 LBC cases, 19 were finally diagnosed with endometrioid carcinoma (EC) through

biopsy or surgical resection, including 6 with G1, 10 with G2, and 3 with G3 grades. The other four cases consisted of one each of dedifferentiated carcinomas (no. 6), mixed EC/clear cell carcinoma (no. 3), carcinosarcoma (no. 4), and large cell neuroendocrine carcinoma (no. 8). From LBC-based NGS, all cancers except for two cases (nos. 10 and 21) showed nearly common mutation profiles, including *PTEN, CTNNB1, PIK3CA,* and *PIK3R1*, which were nearly identical to those obtained from FFPE tissues. The MSI scores were not calculated in three cases (nos. 3, 14, and 21) because of low tumor fraction of 25% or less. Finally, NGS was successful in 19 out of 23 cases using LBC specimens and in all 23 cases except for one case (no. 12) from FFPE specimens.

Evaluation of TMB and MSI status, and WHO molecular classification

The IHC results, TMB and MSI status, and WHO molecular classification are summarized in Table 2. The genomic profiles, TMB and MSI, and WHO molecular classification were compared between LBC and FFPE using 18 cases, excluding four unsuccessful LBC cases (nos. 3, 10, 14, and 21) and one unsuccessful FFPE case (no. 12). All 18 cases showed identical results of TMB and MSI status between LBC and FFPE specimens. Six MMR-deficient cases (nos. 1, 2, 5, 9, 16, and 18) showed TMB-H and MSI-H; among them, four cases harbored mutations in some of *MMR* genes. Three cases (nos. 11, 13, and 23) with pathogenic *POLE* mutations exhibited TMB-UH but

low MSI scores. Case 16 had a *POLE* mutation with a variant of unknown significance was evaluated as TMB-H and MSI-H. Although the precise genomic mutation profiles were not exactly corresponding, the molecular classification of endometrial cancers proposed by the WHO system [20] was well matched between FFPE-based and LBC-based NGS analyses, in which three *POLE* mutations, six MMR-d, five *TP53* mutations, and four NSMP types were classified (Table S2).

Discussion

The present study clearly demonstrated that residual LBC specimens potentially serve as a reliable source for rapid genomic analysis in the diagnosis of endometrial cancer, in combination with pathological examinations. Since good quality DNA can be obtained from preserved LBC specimens, they could be successfully applied for NGS. [16, 17] However, the most important factor for the successful NGS analysis using cytology specimens is the number of tumor cells and input DNA.[7, 25] In this study, when input tumor cells were greater than approximately 10,000 and tumor fraction was greater than 50%, resulting in more than 17 ng of input DNA (17 cases), successful detection of mutations and analysis and calculation of MSI/TMB, which is necessary for WHO molecular classification, were attained.[20]

Since LBC specimens can be directly used for DNA extraction, NGS analysis using LBC specimens would be a time-saving method for cancer genome analysis. In the present study, almost all specimens contained sufficient tumor cells for NGS, although the relative amount required for successful NGS was difficult to evaluate before DNA extraction. In Figure 3, a strategy for routine cancer genomic profiling, cytological, and histopathological diagnosis using LBC and biopsied specimens for endometrial cancer management in a pathology laboratory is presented. As per this strategy, NGS analysis can be started immediately after the cytology sampling, directly exploiting residual LBC specimens, a process through which total workflow will take 5 days. The routine work for FFPE processing and histopathological diagnosis of biopsied tissues takes approximately 3.5 days at Kagoshima University Hospital; therefore, genomic profiling of LBC specimens can be performed at least in the week after pathological diagnosis. The main benefit of preoperative genomic testing is earlier screening for the proof of cancer cell existence, as well as seeking a molecularly targeted drug. Therefore, LBC and biopsied specimens obtained before surgery can be a good source for a rapid preoperative NGS analysis.

Of 21 cases of successful assayed via mutation analysis (except for Case nos. 10 and 21), 9 cases (nos. 2, 3, 11, 13, 14,16,18,22, and 23) did not show perfect match in the

mutations detected from LBC and FFPE specimens. However, the unmatched mutations were not prominent enough to necessitate a change in the interpretation of the genomic profile. In contrast, the TMB and MSI scores were similar in both the LBC and FFPE specimens. Four LBC specimens (nos. 3, 10, 14, and 21) were not suitable for genomic profiling because of their lower tumor fraction. However, since the genomic profiles obtained from LBC specimens corresponded well with those obtained from FFPE specimens, WHO molecular classification was nearly matched between LBC- and FFPE-based NGS, indicating that LBC specimens are also an acceptable source for an appropriate molecular profiling.

In comparison with LBC specimens, conventional smear and CB preparation are indicated to have some advantages for NGS appilication.[11-15] One of the advantages of scraped cells from smears and paraffin sections from CB is that the same cells observed by cytopathologists are subjected to NGS analysis. Another advantage is that it is easy to increase the tumor fraction in smears and CB by scraping and trimming, respectively. In addition, a rapid on-site evaluation to ensure sufficient tumor cell collection is impossible with the LBC procedure. However, one of the notable advantages of using residual LBC specimens is that they can be directly subjected to DNA extraction without any additional procedures or slide sacrifice, unlike CB preparations and cell

scraping.[13, 14] This enables pathologists and gynecologists to gain time, prior to obtaining cancer genome information. The prompt collection of cancer genome information would be beneficial for decision making by gynecologists for subsequent clinical procedures, including the selection of treatment.

In summary, LBC specimens from the endometrium showed several diagnostic gene alterations using a customized cancer gene panel with a 5-day working time. Therefore, residual LBC specimens can be very useful for a rapid molecular testing for endometrial cancer screening.

Availability of data: The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Conflict of interest statement: No conflict of interest declared.

Authors' contributions: Toshiaki Akahane and Yukari Nishida-Kirida contributed as cytotechnologists; Ikumi Kitazono contributed as a cytopathologist; Kazuhiro Tabata contributed as a pathologist; Toshiaki Akahane and Tomomi Yamaguchi analyzed and

interpreted the sequencing data; Yusuke Kobayashi and Shintaro Yanazume summarized the clinical and pathological data; Akihide Tanimoto and Hiroaki Kobayashi organized the study design, and article writing; and all authors read and approved the final manuscript.

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Figure Legend

Figure 1. Workflow of NGS analysis from LBC specimens

The workflow of NGS analysis from residual LBC specimens was presented, starting from DNA extraction, DNA quality check, preparation of NGS library and quality check, NGS run, and gene profiling. The turnaround time was four days.

Figure 2. Representative microphotograph of endometrial LBC

A) LBC specimens contained tumor cells of endometrioid carcinoma in the background of inflammatory cells (Case no. 3, Papanicolaou staining, 200× magnification). The tumor cell number and tumor fraction applied for NGS were counted as 6,328 and 13%, respectively. MSI score was not successfully analyzed due to the low tumor fraction. **B)** Endometrial LBC specimen (Case no. 16) with cell count of 121,318 and tumor fraction of 57 %, that showed successful genomic analysis (Papanicolaou staining, 200× magnification). **C)** LBC specimen (Case no. 21) with cell count of 9,644 and tumor fraction of 15 %, that showed unsuccessful genomic analysis (Papanicolaou staining, 200× magnification).

Figure 3. A proposed strategy for a routine work involving genomic analysis from LBC specimens

A strategy for routine cancer genomic profiling, cytological, and histopathological diagnosis using LBC and tissue specimens in a pathology laboratory is shown. The total work flow time was 5 days, and the turnaround time for histopathological diagnosis was 3.5 days.