Towards less invasive molecular diagnostics for endometrial cancer: massively parallel sequencing of endometrial lavage specimens in women attending for an office hysteroscopy

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Abstract
We aimed to detect endometrial cancer (EC)–associated mutations in endometrial lavage specimens collected in an office setting and to compare the detected mutations with those identified in tissue samples. Participants included 16 women attending for an office hysteroscopy because of suspected EC between July 2020 and October 2021. Massively parallel sequencing was conducted using the targeted 72 cancer‑associated genes. Endometrial lavage specimens, endometrial tissue samples, and blood samples were simultaneously sequenced to establish the concordance of genetic alterations. In this study, the vast majority of EC‑associated mutations identified in lavage samples ($R^2 = 0.948$) were identical to those detected in endometrial tissues. Of the 13 patients with EC, 12 (92.3%) had at least one mutation identified in endometrial lavage samples. Notably, no mutations in lavage samples were identified in the two patients with a previous history of EC but no actual endometrial lesions, supporting a high negative predictive value of the test. A patient previously diagnosed with EC and with current evidence of atypical hyperplasia showed persisting $PTEN$, $PIK3R1$, and $KRAS$ mutations in her endometrial lavage specimen. $PTEN$ was the most commonly mutated gene, followed by $PIK3R1$, $ARID1A$, $PIK3CA$, $CTNNB1$, and $KRAS$. In conclusions, our study provides pilot evidence on the actionability of uterine lavage samples sequencing to detect EC‑associated mutations in women with suspected endometrial lesions. In a precision medicine framework, the high mutational concordance between uterine lavage samples and tissue specimens may help inform less invasive diagnostic protocols and the need for ongoing surveillance in patients with EC who wished for fertility‑preserving treatment.

Key messages
- Sequencing of uterine lavage samples collected by office hysteroscopy is feasible.
- Most EC mutations identified in lavage were identical to endometrial tissues.
- Sequencing of uterine lavage samples may help inform diagnostic protocols for EC.
- This approach can be used for recurrence surveillance in patients with EC.

Keywords Endometrial cancer · Uterine lavage · Biopsy · Diagnostics · Surveillance
Background

Despite decades of intense scientific exploration, endometrial cancer (EC) remains a major public health concern with enormous costs to patients and their families, especially when women of reproductive age are affected [1]. Estimates derived from epidemiological data have shown that the incidence rate of EC for Taiwanese women has been rising from 11.96 cases per 100,000 person in 2012 to 15.11 cases per 100,000 person in 2017 [2, 3]. Even more worryingly, a 51.7% increase in the number of diagnosed cases (from 290 to 440) has been reported for fertile women aged between 30 and 44 years over the same period [2, 3]. While total hysterectomy with bilateral salpingo-oophorectomy remains the mainstay of treatment for resectable EC [1], several fertility-preserving treatment (FPT) options are increasingly available for younger women diagnosed with atypical endometrial hyperplasia or well-differentiated endometrioid EC confined to the endometrium [4–9]. However, disease recurrences in women who wish to preserve their fertility may ultimately undermine therapeutic efforts and result in adverse long-term outcomes. In this scenario, strict follow-up protocols consisting of serial endometrial investigations are generally implemented following conservative treatment [1, 10].

The gold standard for diagnosis of EC remains histological assessment via traditional hysteroscopy under anesthesia [11, 12]. However, because of the cost- and access-to-care barriers and potential morbidity associated with the traditional approach, the development of alternative diagnostic procedures is gaining momentum [11]. In recent years, the availability of “see and treat” operative hysteroscopy performed in an office setting has emerged as a suitable and effective option for EC diagnosis and surveillance in many women [13, 14]. In conjunction with hysterectomy in an office setting, the collection of uterine lavage samples is established as a safe and fast procedure, more acceptable to patients than endometrial biopsy. Therefore, cytological analysis of the uterine lavage fluid had been proposed as a potential screening method for diagnosing EC or detecting tumor recurrence [15]. Unfortunately, this approach is not only laborious and time-consuming but also caution with interpretation of results given the concern for variability. Backed up by advances in high-throughput genetic technologies, the identification of cancer-associated mutations in endometrial lavage samples has the potential to provide clues to a timely and less invasive molecular diagnostic test suitable for EC diagnosis and surveillance [16, 17].

The aim of this study was to apply massively parallel sequencing of endometrial lavage specimens collected in an office setting to detect EC-associated mutations in women with suspected endometrial lesions to help inform diagnostic protocols and the need for ongoing surveillance who wished to preserve fertility. Endometrial tissue samples and blood specimens were simultaneously sequenced to establish the concordance of genetic alterations in different biological matrices.

Methods

Participants

Between July 2020 and October 2021, 16 women who met the inclusion criteria were prospectively invited to participate in this study. Eligibility criteria were as follows: (1) attendance for an office hysteroscopy; (2) recent abnormal uterine bleeding due to a suspected endometrial lesion [18]; (3) previous history of International Federation of Gynecology and Obstetrics (FIGO) stage I EC managed using FPT; and (4) willingness to provide signed informed consent. Women were excluded if they were unwilling to participate in the study or there was suspicion of endometritis [19]. Three participants had previously undergone FPT for stage I EC and were therefore attending office hysteroscopy for surveillance purposes. One woman had a past history of breast invasive ductal carcinoma. The remaining 12 women had no previous history of cancer. In all cases, the final diagnosis was achieved by pathological analysis of endometrial biopsies collected during office hysteroscopy. The Institutional Review Board approval of the Chang Gung Memorial Hospital (identifier: 202001329B0) granted ethical approval to process and analyze all clinical/demographic data and biological samples. Written informed consent was obtained from the patients.

Collection of endometrial lavage samples

Office hysteroscopy was performed with the vaginoscopic technique without the use of a vaginal speculum and/or a tenaculum [12, 20]. Before endometrial biopsies, normal saline was instilled to provide distension and irrigation of the uterine cavity with 25 mL collected. Endometrial lavage samples were obtained with a continuous-flow rigid hysteroscopy system (sheath diameter: 4 mm, RICHARD WOLF, GmbH, Knittlingen, Germany) and centrifuged at 3200 g for 20 min at 4 °C within 2 h of collection. Cell pellets were washed with red blood cell lysis solution and incubated at room temperature for 5 min. After removal of the supernatant, cell pellets obtained from endometrial lavage were transferred to polypropylene tubes and stored – 80 °C until DNA extraction [17].
Collection of endometrial tissue and blood specimens

Endometrial biopsies were collected from all participants during office hysteroscopy and processed to obtain formalin-fixed paraffin-embedded (FFPE) tissue blocks. Additionally, cancer tissue FFPE specimens were retrieved from the archives for the three women with a previous diagnosis of stage I EC. Blood samples were collected from all participants to confirm whether the variants identified in other biological matrices (i.e., endometrial lavage samples, endometrial biopsies, and FFPE specimens) were germline or somatic.

DNA extraction

Genomic DNA was extracted with the use of standard protocols [21–23] from endometrial lavage samples (all women), endometrial biopsies (all women), previous EC FFPE specimens (three women with a previous diagnosis of stage I EC), and blood samples (all women). DNA concentrations and integrity were measured with a Quant-iT dsDNA high-sensitivity assay kit (Invitrogen, Carlsbad, CA, USA) and a fragment analyzer (Advanced Analytical Technologies, Ankeny, IA, USA), respectively.

Massively parallel sequencing and mutation analysis

A polymerase chain reaction (PCR)-based strategy was adopted to enrich all coding exon sequences of the targeted genes. In order to identify EC-associated mutations in the four biological matrices (endometrial lavage samples, endometrial biopsies, FFPE specimens, and blood samples), we undertook massively parallel sequencing (2 × 150 bp paired-end run) on a NextSeq 500 Sequencing System (Illumina, San Diego, CA, USA) using the QIAseq targeted DNA panel DHS-005z that targeted 72 cancer-associated genes (Supplementary Table 1). Uniformity of coverage for tumor samples was set at 95%. All variants with an allele frequency of less than 1% in endometrial lavage samples [17] and 5% in all other biological matrices [24] were filtered out. Raw data generated from the sequencer were mapped to the reference human genome hg19. Each variant was annotated using the following packages: COSMIC (v. 81), gnomAD genome and exome databases, East Asian population (ExAC_EAS), ClinVar database [25], and 1000 Genomes Phase 3. In our study, the total cost for specimen preparation and sequencing was 250 US dollars per sample.

Results

Pathological diagnosis

Table 1 summarizes the final pathological diagnosis of endometrial biopsies in the 16 women who had undergone office hysteroscopy and collection of endometrial lavage samples. Of them, 12 had a de novo diagnosis of endometrioid carcinoma. One participant with a known history of breast invasive ductal carcinoma had a newly detected endometrial metastasis. The remaining three patients who had previously undergone FPT of stage I EC received hysteroscopy for surveillance. The clinical management of the study participants is described in Table 1.

Mutation concordance analysis

Figure 1 depicts all somatic mutations identified in endometrial lavage samples and endometrial biopsies for the 13 women with newly detected uterine lesions (endometrioid carcinoma, n = 12; breast ductal carcinoma metastatic to the endometrium, n = 1). The mutations identified in endometrial lavage samples, simultaneous endometrial biopsies, and previous EC FFPE specimens (for the three women who had previously undergone FPT for stage I EC) are presented in Fig. 2. Detailed mutation data are presented in Supplementary Table 2. A summary of the concordance between mutations identified in endometrial lavage samples and endometrial biopsies in the 16 study participants is provided in Table 2. A total of 74 (allele frequency > 1%) and 78 (allele frequency > 5%) mutations in EC-associated genes were detected in endometrial lavage samples and tissue specimens, respectively. Specifically, 37 missense, 32 nonsense (8 premature stop codon, 24 frameshift), and 5 splice site mutations were identified in lavage samples while 41 missense, 33 nonsense (7 premature stop codon, 26 frameshift), and 4 splice site mutations in tissues. The median allele frequency was 7.44% (range: 2.46–42.12%) and 23.34% (5.02–75.78%) for mutations in endometrial lavage samples and tissue specimens, respectively. Mutations identified in endometrial lavage samples and tissue specimens were highly concordant ($R^2 = 0.948$, Pearson’s correlation coefficient). Additionally, a concordance of the mutation profile was observed in a patient-based analysis (Table 2).

Somatic mutations in endometrial lavage samples obtained from women with newly detected uterine lesions

Of the 13 women with newly detected uterine lesions (endometrioid carcinoma, n = 12; breast ductal carcinoma metastatic to the endometrium, n = 1), 12 (92.3%) harbored
at least one mutation in endometrial lavage samples. However, mutations in tissue specimens were identified in all of the 13 cases (Fig. 1). The concordance of mutation patterns between endometrial lavage samples and biopsy specimens is reported in Supplementary Table 3. A total of 71 (allele frequency > 1%) and 68 (allele frequency > 5%) mutations in EC-associated genes were detected in endometrial lavage samples and tissue specimens, respectively. The most commonly mutated gene was PTEN (69.2%; 9/13), followed by PIK3CA, ARID1A, PIK3CA, CTNNB1, and KRAS.

On analyzing the 10 cases with early-staged EC who had undergone definitive treatment, patients #01 and #04 were classified as harboring hypermutated EC. Specifically, patient #01 had mutations in 12 genes, whereas patient #04 had 11 mutated genes in lavage samples. Patients #05 and #03 had mutations in 6 and 5 genes, respectively. Notably, some mutations (e.g., PIK3CA p.G118D and FBXW7 p.R387L) identified in patient #05 were evident in endometrial lavage samples but not in the corresponding tissue specimens (Supplementary Table 2). Patient #08 had four mutated genes, whereas four cases (#02, #06, #07, and #10) had mutations in three genes. No endometrial lavage sample mutations were identified in patient #9.

Patient #11 was diagnosed with stage IIIA endometrioid carcinoma, and four somatic mutations were identified in both endometrial lavage samples and the corresponding tissue specimens. However, the SETD2 p.T532I mutation—which showed an allele frequency of 5.29% in the endometrial tissue specimen—was not identified in the endometrial lavage sample. Patient #12 had stage IIIC1 endometrioid carcinoma, and three somatic mutations were identified in both endometrial lavage samples and the corresponding tissue specimens. On analyzing the validity of this method in different clinical scenarios, we included patient #13 who had a breast ductal carcinoma metastatic to the endometrium. Notably, we were able to identify the PKHD1 p.M3273R mutation in the endometrial lavage sample (allele frequency: 6.30%). Another MET mutation was shared between the endometrial lavage sample and the corresponding tissue specimen.

Somatic mutations in endometrial lavage samples obtained from women who had previously undergone fertility-preserving treatment of endometrial carcinoma

The results of pathology in the three women who had previously undergone FPT of stage I EC revealed progestin effect without residual tumor cells in two cases and atypical hyperplasia in one case. Patient #14 (diagnosed with progestin effect due to previous progestin therapy) had PTEN and PIK3CA mutations in archived FFPE specimens of the known EC (Fig. 2) but—as expected based on the current diagnosis—no mutation was detected in endometrial lavage samples. Similarly, patient #15 (diagnosed with progestin effect due to previous progestin therapy) had PTEN and CTNNB1 mutations in archived FFPE specimens of the known EC but no mutation in endometrial lavage samples. Finally, patient
#16 (diagnosed with atypical hyperplasia) carried PTEN, PIK3R1, and KRAS mutations in archived FFPE specimens of the known EC. The same mutations were identified in both biopsy specimens collected during office hysteroscopy and endometrial lavage samples.

**Discussion**

Compared with traditional endometrial biopsy, endometrial lavage-based tumor biomarkers have significant advantages—including time and cost-efficiency, increased patient acceptance, and reduced invasiveness. In this study conducted in 16 women attending for an office hysteroscopy for suspected EC, we have provided proof-of-concept evidence that mutation analysis of endometrial lavage samples is feasible. Importantly, the vast majority of EC-associated mutations identified in lavage samples ($R^2 = 0.948$) were identical to those detected in tissue specimens. While no mutations in endometrial lavage samples were detected in the two patients with previous EC (final pathological diagnosis: progestin effect), persistent PTEN, PIK3R1, and KRAS mutations were identified in the patient with a known history of EC who had current evidence of atypical hyperplasia. Collectively, these results suggest that genetic profiling of endometrial lavage fluid obtained during office hysteroscopy is a promising molecular approach to support the diagnosis and ongoing surveillance of EC.

While the mutational concordance was generally high in our study, a difference in median allele frequency was observed between endometrial lavage samples (7.44) and tissue specimens (23.34). In this scenario, different thresholds were used to filter out variants (less than 1% in endometrial lavage samples [17] and 5% in tissue samples [24]). Notably, we found sporadic discordances between endometrial lavage and tissue mutational profiling. In the case of mutations detected in endometrial lavage samples but not in endometrial specimens (e.g., patient #05; PIK3CA p.G118D mutation; Supplementary Table 2), the discrepancy may be attributed to intra-tumoral heterogeneity. The opposite scenario (i.e., mutations detected in endometrial specimens but not in endometrial lavage samples) can occur for somatic alterations at low variant allele frequency (e.g., patient #11; SETD2 p.T532I mutation, allele frequency: 5.29%);
Supplementary Table 2). While not observed in the current study, the occurrence of somatic mutations in the normal endometrium [26] may be another contributing factor. A longitudinal follow-up study of women with discordances between endometrial lavage and tissue mutational profiling is necessary to determine the clinical value of serial mutation analyses in this patient group.

In our cohort, PTEN—a tumor suppressor gene that encodes a dual-specificity phosphatase—was the most commonly mutated gene, followed by PIK3R1, ARID1A, PIK3CA, CTNNB1, and KRAS. Interestingly, these findings are in line with those of Martignetti et al. [27]. These authors have suggested that the detection of PTEN mutations in uterine lavage fluid obtained from women undergoing hysteroscopy may serve as a biomarker for detecting EC recurrence [27]. These findings provide preliminary evidence that uterine lavage fluid-based biomarkers included in the QIAseq targeted DNA panel DHS-005z is reliable for the screening of EC. Importantly, hysteroscopy allows direct visualization of the endometrial cavity during lavage and avoids potential sampling errors that may occur with the use of other techniques (e.g., Pap smear, Tao brush, and Pipelle devices) [28, 29].

While still being applied mainly in research settings, massively parallel sequencing of endometrial lavage specimens holds promise to complement endometrial biopsy for providing a more comprehensive depiction of the disease status during the follow-up of patients who had undergone FPT. In addition, endometrial biopsies targeting the areas of clinical suspicion are not without challenges in presence of small foci of residual disease—which are not uncommon in patients treated with FPT. As sampling and/or interpretation errors may occasionally lead to false-negative results, this technique can be helpful to reduce the rates of false-negative findings on endometrial biopsies as well as in the identification of subclonal mutations that might be missed when endometrial biopsy specimens are analyzed. If independently validated, the molecular alterations identified in our study can also be valuable to reduce the time needed to decide on any adjuvant therapy.

In addition to the traditional histological classification, a molecular phenotyping of EC into four subtypes (POLE-mutated, microsatellite instability, copy-number low, and copy-number high) has been recently proposed [30]. It is also worth noting that molecular markers are currently being adopted by the European Society for Medical Oncology.
The financial burden of next-generation sequencing has declined significantly over the last few years [34]. However, the total cost for specimen preparation and sequencing in this study remained significant (250 US dollars per sample). While financial and technical barriers still preclude the clinical application of the approach described in our study in a routine fashion, this technique will likely play an increasingly prominent role in predicting outcomes and informing clinical management in the coming years when further cost reductions are anticipated. Moreover, the integration of massively parallel sequencing data into clinical care is expected to enable personalized treatment and clinical care of endometrial cancer in the near future.

There are limitations to our study. First, its pilot nature and the small sample size may have limited the external validity of the results, and, for that reason, larger cohorts are required to confirm our data. Currently, our findings should

| Gene     | Endometrial lavage fluid, number of mutations (frequency > 1%) | Endometrial biopsies, number of mutations (frequency > 5%) | Mutation concordance |
|----------|---------------------------------------------------------------|------------------------------------------------------------|----------------------|
|          | Number of mutations | Number of patients | Number of mutations | Number of patients |
| PTEN     | 15                  | 17                | 13                  | 10                  |
| PIK3R1   | 9                   | 8                 | 7                   | 7                   |
| ARID1A   | 11                  | 9                 | 7                   | 5                   |
| PIK3CA   | 5                   | 6                 | 4                   | 4                   |
| CTNNB1   | 3                   | 3                 | 3                   | 3                   |
| KRAS     | 3                   |                   | 3                   | 3                   |
| AKT1     | 1                   | 1                 | 1                   | 1                   |
| AMER1    | 1                   | 1                 | 1                   | 1                   |
| APC      | 1                   |                   | 1                   | 1                   |
| BAI3     | 1                   |                   | 1                   | 1                   |
| CDKN2A   | 1                   |                   | 1                   | 1                   |
| CREBBP   | 2                   | 2                 | 1                   | 1                   |
| ERBB2    | 1                   |                   | 1                   | 1                   |
| FBXW7    | 2                   | 1                 | 1                   | 1                   |
| FGFR2    | 2                   | 2                 | 1                   | 1                   |
| KEAP1    | 2                   |                   | 1                   | 1                   |
| KMT2D    | 1                   |                   | 1                   | 1                   |
| MAP2K1   | 1                   |                   | 1                   | 1                   |
| MET      | 1                   |                   | 1                   | 1                   |
| MLH1     | 1                   |                   | 1                   | 1                   |
| NFE2L2   | 1                   |                   | 1                   | 1                   |
| ROS1     | 1                   |                   | 1                   | 1                   |
| SETD2    | 1                   | 2                 | 1                   | 1                   |
| SMAD4    | 1                   |                   | 1                   | 1                   |
| SMARCA4  | 1                   | 2                 | 1                   | 1                   |
| U2AF1    | 1                   |                   | 1                   | 1                   |
| EGFR     | 1                   | 0                 | 0                   | 0                   |
| PKHD1    | 1                   | 0                 | 0                   | 0                   |
| RET      | 1                   | 0                 | 0                   | 0                   |
| TNFAIP3  | 1                   | 0                 | 0                   | 0                   |
| TP53     | 0                   | 1                 | 0                   | 0                   |
be considered hypothesis-generating. We nonetheless believe that the identification of the molecular underpinnings of early-stage EC—especially in a subset of patients who had undergone FPT—is of interest and may prompt additional research in the field. Second, this study was not designed to examine transcervical resection samples obtained through cervical dilatation performed in an operating room, and our exclusive focus on office hysteroscopy resulted in a slow patient enrollment. A standardization of the procedures used for endometrial lavage fluid collection (e.g., application of a continuous flow system) in an office setting will hopefully facilitate a broader implementation of this approach. Finally, it would have been interesting to include cytological analysis of uterine lavage samples [16].

Conclusions

Our study provides pilot evidence on the actionability of uterine lavage samples sequencing to assess the presence of mutations in women with EC. In a precision medicine framework, the high mutational concordance between uterine lavage samples and tissue specimens may help inform less invasive diagnostic protocols and the need for ongoing surveillance in patients with EC who wish to preserve fertility.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00109-022-02239-7.

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Author contribution A.C. and R.C.W.: study concept and design. K.Y.W., C.H.W., Y.S.L., Y.S.T., H.J.H., C.H.L., T.C.C., and A.S.C.: data collection and interpretation. R.C.W.: pathological examinations. A.C. C.Y.L., and R.C.W.: manuscript drafting. A.C. and R.C.W.: critical revision of the manuscript for impact intellectual content. The manuscript has been read and approved by all authors.

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Data availability All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethical approval The Institutional Review Board approval of the Chang Gung Memorial Hospital (identifier: 202001329B0) granted ethical approval to process and analyze all clinical/demographic data and biological samples. Written informed consent was obtained from the patients.

Conflict of interest The authors declare no competing interests.

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