Genomic Profiling Using Vaginal Swab and Plasma in Patients with Endometrial Cancer by Deep Sequencing

Namsoo Kim

The Graduate School
Yonsei University
Department of Medicine

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Namsoo Kim

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This certifies that the Dissertation of Namsoo Kim is approved

signature

Thesis Supervisor Jong Rak Choi

signature

Thesis Committee Member Seung-Tae Lee

signature

Thesis Committee Member Hyo Sup Shim

signature

Thesis Committee Member Jung-Yun Lee

signature

Thesis Committee Member Jihye Ha

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ABSTRACT

Genomic Profiling Using Vaginal Swab and Plasma in Patients with Endometrial Cancer by Deep Sequencing

Endometrial cancer is the most common gynecological cancer in the USA and has recently surpassed cervical cancer as the leading gynecological cancer in Korea. Diagnosing it is challenging due to its heterogeneity, and current methods like tissue-based next-generation sequencing (NGS) are invasive and may miss important mutations. This study aimed to explore noninvasive alternatives by analyzing genomic DNA (gDNA) from vaginal swabs and circulating tumor DNA (ctDNA) from plasma to assess their potential for genomic profiling and predicting outcomes in endometrial cancer.

This prospective study involved 191 patients, with both cancer and benign cases. Vaginal swab-based gDNA and plasma-based ctDNA were collected for analysis. NGS targeting 101 genes was used, focusing on common mutations like *PTEN*, *TP53*, and *PIK3CA*. Also, plasma samples were obtained at multiple time points postsurgery to assess the role of plasma-based ctDNA in monitoring recurrence and progression.

The results indicated that vaginal swab-based gDNA exhibited 77.7% sensitivity and 96.6% specificity, making it a useful tool for detecting mutations, especially in early-stage endometrial cancer. *PTEN*, *TP53*, and *PIK3CA* mutations were the most common. This study established a novel classification, comprising only *PTEN* and *TP53* mutations, based on the association of *TP53* mutations with adverse prognosis and *PTEN* mutations with favorable prognosis. Vaginal swab-based gDNA also strongly correlated with prognostic factors like lymphovascular invasion and was more effective than traditional PAP cytology in detecting cancer in negative cytology cases.

Plasma-based ctDNA analysis, although less sensitive in early-stage cancers, was more closely linked to advanced-stage disease, lymphovascular invasion, and recurrence, highlighting its potential for posttreatment monitoring. Cox regression analysis identified both lymphovascular invasion and plasma-based ctDNA positivity as significant prognostic factors. Patients with positive plasma-based ctDNA results had significantly worse outcomes, suggesting plasma-based ctDNA can predict recurrence earlier than conventional imaging methods in some cases.

A novel aspect of this study was the application of both vaginal swab-based gDNA and plasma-based ctDNA through deep sequencing across a large cohort of endometrial cancer patients. These noninvasive techniques allowed us to track genomic alterations over time, providing

insights into tumor progression and resistance. The study also explored cost-efficient alternatives to three full-gene sequencing and three targeting specific mutation hotspot genes, maintaining high diagnostic accuracy while lowering the cost of comprehensive testing.

In conclusion, vaginal swab-based gDNA shows promise for diagnosing and profiling endometrial cancer for identifying key prognostic mutations. Plasma-based ctDNA complements this by providing critical information on tumor burden, recurrence, and prognosis. Combining these methods could improve diagnosis and monitoring for endometrial cancer, although further research is needed to improve accuracy, refine clinical use, optimize sensitivity, and assess their cost-effectiveness in broader clinical settings.

Key words : Endometrial cancer, Vaginal swab-based gDNA, Plasma-based ctDNA, Next-generation sequencing (NGS), *TP53*, *PTEN*, Prognostic factors, Recurrence monitoring, Noninvasive diagnosis

I. INTRODUCTION

1.1. Overview of Endometrial Cancer

Endometrial cancer is a type of cancer that originates from the inner lining of the uterus and is becoming more prevalent worldwide(1). It is the most common gynecological cancer in the USA(2), and recent reports show it has surpassed cervical cancer as the leading gynecological cancer in Korea, ranking sixth among all cancers in women(3). Diagnostic methods include Papanicolaou test (PAP smears), blood markers such as CA-125, and transvaginal ultrasounds for early detection(4). Imaging techniques like computed tomography (CT) and magnetic resonance imaging are also utilized, but the most critical aspect of the workup is the pathologic evaluation of the endometrium via curettage(5). Surgical staging is essential to determining prognosis(6).

1.2. Molecular Diagnosis of Endometrial Cancer

Molecular diagnosis is becoming important for endometrial cancer as more cancers are being sequenced(7). Methods include sequencing cancer tissue from the endometrium, analyzing plasma-based circulating tumor DNA (ctDNA) from whole blood samples, and more recently, analyzing vaginal swab-based genomic DNA (gDNA)(8). Sequencing the cancer tissue from the endometrium is the most invasive method and is efficient for mutation detection(9). However, due to cancer heterogeneity, it may miss some mutations(10). Plasma-based ctDNA analysis is effective for detecting driver mutations in advanced stages endometrial cancer(11), but has an 18% detection rate in early stages(8). Despite the lower detection rate in the early stages, the advantage of plasma-based ctDNA analysis is that it can be collected repeatedly from advanced or recurrent patients, unlike uterine or vaginal tissues that may be removed during surgery. Vaginal swab-based gDNA analysis is a newer, noninvasive method that studies the cancer's molecular profile by detecting cancer cells shed into the cervix.

1.3. ProMisE Classification

Following the use of these methods for molecular profiling, the Proactive Molecular Risk Classifier guideline (ProMisE) algorithm has been proposed by the European Society for Medical Oncology, the European Society for Radiotherapy and Oncology, and the European Society of Gynecological Oncology(12). It categorizes endometrial cancer into the following mismatch repair (MMR), *POLE*, and *TP53* mutation groups: mismatch repair deficient (MMR-D), *POLE* exonuclease domain mutations (*POLE* EDM), p53 abnormal (p53 abn), and p53 wild type (p53 wt). *POLE* is a gene encoding the catalytic subunit of DNA polymerase epsilon, involved in DNA replication and repair. *POLE* mutations are associated with a high mutational burden, leading to an exceptionally high number of somatic mutations and increased immunogenicity, leading to a stronger immune response and better prognosis. In contrast, *TP53* mutations, which are involved

in cell cycle regulation, DNA repair, and apoptosis, are associated with more aggressive endometrial cancer and worse prognosis. Molecular classification using next-generation sequencing (NGS) is sometimes replaced by immunohistochemistry (IHC) staining for practical clinical use.

1.4. Genetic Alterations in Endometrial Cancer

Extensive analyses have been conducted on relevant genes in endometrial cancer. *PTEN* is the most common genetic alteration(13), while *TP53* is linked to poor prognosis(14). Recently, there has been a growing interest in *CTNNB1* mutations, particularly in *TP53*-negative patients(15). *MSH2*, *MSH6*, *PMS2*, and *MLH1* are mismatch repair genes(16). Multiple genes are being investigated in relation to prognosis, emphasizing the need for comprehensive research in this area.

1.5. Objectives of the Study

This study used an integrative approach, combining traditional methods with the following strategies. Instead of using tissue samples, this study analyzed vaginal swab-based gDNA and plasma-based ctDNA, collected noninvasively from vaginal swabs and whole blood, respectively. This prospective study included diverse patient groups, focusing on advanced-stage patients, with plasma-based ctDNA collected at several-month intervals postsurgery. This study conducted NGS to target 101 genes associated with endometrial cancer and considered various patient factors to gain a detailed understanding of the genetic landscape. This study aims (1) to determine if vaginal swab-based gDNA can aid in genomic profiling, (2) to investigate the potential of vaginal swab-based gDNA for identifying prognostic factors in endometrial cancer, and (3) to assess the use of plasma-based ctDNA monitoring for effective patient follow-up.

II. MATERIALS AND METHODS

2.1. Patient Recruitment

Patients with endometrial cancer were enrolled in this study between January 2021 and November 2022 (ClinicalTrials.gov Identifier: NCT05504161). Only adult patients with confirmed endometrial cancer were included, whereas patients with ovarian or cervical cancer were excluded. Additionally, recured and rerecured patients were included for ctDNA testing as they had already undergone total hysterectomy. As a control group, patients with noncancerous endometrial conditions (like endometrial hyperplasia, endometrial polyps, atypical hyperplasia (AH), and endometrial intraepithelial neoplasia (EIN)) undergoing resectoscope-guided procedures were also recruited. Preoperative PAP smear and postoperative histological results were analyzed for all patients.

Vaginal swabs were collected using a Cervex-Brush (Rovers Medical Device, Noord-Brabant, Netherlands) after the patient entered the operating room, without using a speculum. Whole blood samples were drawn in ctDNA-specific Dxtube bottles (Dxome Co. Ltd., Seongnam, Gyeonggi-do, Republic of Korea) immediately upon the patient's entry into the operating room. In addition, whole blood samples were collected at several-month intervals postsurgery where feasible. The study protocol adhered to the ethical principles outlined in the Declaration of Helsinki and was approved by the Institutional Review Board of Severance Hospital, Seoul, Korea (approval no. 4-2020-1265).

2.2. Acquisition of gDNA, ctDNA, and NGS

For vaginal swab-based gDNA analysis, the swabs were processed in a 50-mL conical tube containing saline and centrifuged at 1900 *g* for 10 min to remove the pellet, leaving 1 mL of the supernatant for further experiments. Plasma-based ctDNA was extracted from whole blood separated from the Dxtube within 4 days of collection. After a 15-min centrifugation at 1900 *g*, both the supernatant and buffy coat were collected to distinguish germline mutations. The supernatant was stored at 80°C for future batch sequencing once sufficient samples were accumulated.

To isolate gDNA from the vaginal swab samples, 0.2 mL of the supernatant was processed using the QIAamp DNA Mini Kit (51306, QIAGEN, Hilden, Germany). The buffy coat from whole blood samples was processed using the same kit. Plasma-based ctDNA was isolated using the Magnetic Circulating DNA Maxi Reagent (Dxome) and eluted in 100 μL of elution buffer. The extracted DNA was then sheared to an average size of 200–250 base pairs using the D1000 ScreenTape system (Agilent, CA, USA) for size and quantity measurement.

For NGS, library preparation was performed using the DxLiquid NGS system for Illumina (Cat No. LP01096, Dxome), with 100–200 ng of gDNA for vaginal swab samples and 5–30 ng of ctDNA for plasma samples. The number of polymerase chain reaction (PCR) cycles varied between 7 and 13, depending on the input amount.

Sequencing targeted 101 genes (Table 1) associated with endometrial cancer using the DxLiquid Pan100 kit (Cat no. DL-AO1001024, Dxome). It was performed using Illumina instruments (Novaseq or Nextseq2000) with the position indexing sequencing (PiSeq) algorithm, which detects true somatic variants using positional data from aligned uncollapsed reads generated through duplex sequencing(17). Positional information is presented as a molecular "barcode," where true-positive variants are identified in all aligned reads from the same source, while false positives are not. Variants were classified into four tiers based on the Association for Molecular Pathology with liaison representation from the American College of Medical Genetics and Genomics, the American Society of Clinical Oncology, and the College of American Pathologists(18). Databases like OncoKB, cBioPortal, and My Cancer Genome were used for classification(19-21). Tier IV variants (high likelihood of benignity) excluded, and all other variants were visually confirmed using the Integrated Genomics Viewer(22). Samples were classified as "positive" for detected tier I or II variants, "borderline" for detected tier III variants only, and "negative" if no variants from tier I, II, or III were detected. Sequencing also included analysis of 50 microsatellite instability (MSI) target sites (Table 2) with raw data of bidirectional sequence reads (nBi).

Table 1. Targeted 101 genes associated with endometrial cancer

AKT1	*CDK4*	*EZH2*	*KIT*	*PDGFRA*
AKT2	*CDK6*	*FANCL*	*KRAS*	*PDGFRB*
AKT3	*CDKN1A*	*FBXW7*	*MAP2K1*	*PIK3CA*
ALK	*CDKN1B*	*FGFR1*	*MAP2K2*	*PMS2*
APC	*CDKN2A*	*FGFR2*	*MCL1*	*POLD1*
AR	*CDKN2B*	*FGFR3*	*MDM2*	*POLE*
ARAF	*CHEK1*	*FGFR4*	*MET*	*PTCH1*
ARID1A	*CHEK2*	*FLCN*	*MLH1*	*PTEN*
ATM	*CRKL*	*FLT3*	*MSH2*	*RAD51B*
ATR	*CTNNB1*	*GNA11*	*MSH6*	*RAD54L*
BARD1	*DDR2*	*GNAQ*	*MTOR*	*RAF1*
BRAF	*DNMT3A*	*GNAS*	*MYC*	*RB1*
BRCA1	*EGFR*	*HNF1A*	*MYCL*	*RET*
BRCA2	*EPCAM*	*HRAS*	*MYCN*	*SMAD4*
BRIP1	*EPHA3*	*IDH1*	*NF1*	*SMO*
CCND1	*ERBB2*	*IDH2*	*NF2*	*STK11*
CCNE1	*ERBB3*	*IGF1R*	*NOTCH1*	*TERT*
CD274	*ERBB4*	*JAK2*	*NOTCH2*	*TP53*
CDH1	*ERCC2*	*JAK3*	*NRAS*	*TSC1*
CDK12	*ESR1*	*KDR*	*PALB2*	*TSC2*
VHL				

Table 2. Target sites of microsatellite instability

chr1:162736821-162736822	chr3:142231062-142231063	chr7:6038620-6038621	chr10:79797660-79797661	chr14:68944343-68944344
chr1:23689786-23689787	chr3:170784256-170784257	chr7:6037057-6037058	chr11:108188266-108188267	chr14:75282873-75282874
chr2:212578379-212578380	chr4:153268227-153268228	chr7:6036847-6036848	chr11:108114661-108114662	chr14:68934755-68934756
chr2:47635523-47635524	chr4:55976947-55976948	chr7:116409675-116409676	chr11:108100105-108100106	chr14:23652346-23652347
chr2:215657182-215657183	chr4:55131001-55131002	chr7:83021799-83021800	chr11:108141955-108141956	chr15:83808031-83808032
chr2:48032740-48032741	chr4:99801986-99801987	chr8:62496421-62496422	chr11:60296728-60296729	chr17:29508819-29508820
chr2:219614190-219614191	chr4:55598211-55598212	chr9:135781935-135781936	chr12:45833430-45833431	chr17:40647390-40647391
chr2:47641559-47641560	chr5:78671727-78671728	chr9:80343587-80343588	chr13:49039094-49039095	chr17:29559061-29559062
chr2:95849361-95849362	chr6:142691950-142691951	chr9:135773000-135773001	chr13:48954159-48954160	chr18:48584855-48584856
chr2:39564893-39564894	chr7:116381121-116381122	chr10:43595836-43595837	chr13:21732028-21732029	chr22:30051705-30051706

2.3. Collection of Clinical Variables and the Immunohistochemistry Profile

Endometrial samples were obtained via hysterectomy, resectoscope procedures, or dilation and curettage. A board-certified pathologist reviewed and evaluated these samples, focusing on the pathology, tumor grade, size, depth, and lymphovascular invasion. Cancer staging was determined according to the FIGO system(6).

For a subset of endometrial cancer patients, immunohistochemistry (IHC) was done to profile mismatch repair proteins (p53, *MSH2*, *MSH6*, *PMS2*, and *MLH1*) using formalin-fixed, paraffin-embedded tissue specimens. IHC was performed using a Ventana Discovery XT Automated Slide Stainer (Ventana Medical System, Tucson, AZ, USA) after deparaffinizing with xylene and rehydrating with a graded alcohol series. Cell Conditioning Buffer 1 (citrate buffer, pH 6.0; Ventana Medical System) was used for antigen retrieval. The slides were incubated with primary antibodies targeting *MLH1* (dilution 1:50, BD Biosciences, San Jose, CA, USA), *MSH2* (dilution 1:200, BD Biosciences), *MSH6* (dilution 1:100, Cell Marque Corporation, Rocklin, CA, USA), *PMS2* (dilution 1:40, Cell Marque), and p53 (dilution 1:50, Dako, CA, USA). The stained slides were scored and interpreted by an expert pathologist.

2.4. Statistical Analysis

Statistical analysis was performed using Python and R. Data compilation and analysis were conducted using the Python library “pandas,” while genomic data from variant calling files were processed using the R package “maftools”(23). Categorical variables were analyzed using Fisher’s exact test or chi-square test, and continuous variables were compared using Student’s *t*-test. For matched samples, the McNemar test with continuity correction was used. Mutations were mapped onto protein structures using the ProteinPaint tool(24). Survival rates and factors were analyzed using Cox regression and Kaplan–Meier analysis. $p < 0.05$ was considered statistically significant.

III. Results

3.1. Overview of Patients and Sample Characteristics

A total of 191 patients participated in the study: 42 with benign endometrial conditions and 149 with endometrial cancer (Table 3). Of these, 44 patients provided samples only for vaginal swab-based gDNA analysis, with 19 from the benign group and 25 from the cancer group. Similarly, plasma-based ctDNA was collected from 50 patients, including 13 benign cases and 37 cancer cases. Additionally, both vaginal swab-based gDNA and plasma-based ctDNA were collected from 97 patients, with 10 benign cases and 87 cancer cases.

A total of 386 samples, including vaginal swab-based gDNA and plasma-based ctDNA, were collected from the 191 patients (Table 4). This included 141 vaginal swab-based gDNA samples (29 benign or AH/EIN samples and 112 endometrial cancer samples) and 145 plasma-based ctDNA samples (22 from benign or AH/EIN samples and 223 endometrial cancer samples). A total of 44 patients provided more than one plasma sample.

Table 3. Distribution of sample collection by patient group

Patient Group	Vaginal Swab-based gDNA only	Plasma-based ctDNA only	Both	Total
Benign	19	13	10	42
Cancer	25	37	87	149

Table 4. Sample status and number of collections

Sample Type	Status		*n*
Vaginal Swab-based gDNA	Benign		29
	Cancer		112
Total			141
Plasma-based ctDNA	Benign		23
	Cancer	1st	124
		2nd	44
		3rd	32
		4th	15
		5th	7
		6th	2
Total			245
Overall Total			386

3.2. Analysis of Vaginal Swab-based gDNA

This section describes the 141 patients from whom vaginal swab-based gDNA was collected. Their demographic features are detailed in Table 5, with an average age of 53.3 years. Of these, 29 had benign or AH/EIN conditions. Among the 112 endometrial cancer patients, 63 (56.3%) were at stage IA, and 17 (15.2%) were in advanced stages (stage III or IV). Endometrioid cancer was the most common, accountin for 96 cases (85.7%).

The results of vaginal swab-based gDNA were analyzed based on stage, cytology, lymphovascular invasion, ctDNA, and prognosis (Table 6). Among the 29 patients with negative or borderline results in benign or AH/EIN conditions, 28 (96.6%) were observed while 87 out of 112 patients with positive results had endometrial cancer (77.7%). Only 4 out of 49 patients from stage IB to IV did not show positive results, while 15 out of 17 patients with advanced stages (stage III or IV) exhibited positive results (88.2%). Among 74 patients without lymphovascular invasion, 54 (73.0%) were positive, while 32 out of 35 patients with lymphovascular invasion (91.4%) were positive. Regarding prognosis, 69 out of 121 patients with no evidence of disease (NED) were positive (57.0%), whereas 16 out of 18 patients who experienced recurrence or death were positive (88.9%).

Results comparing conventional PAP cytology and plasma-based ctDNA were analyzed specifically for endometrial cancer patients (Table 7). Among 10 patients with malignancy detected by conventional PAP cytology, 9 tested positive for gDNA. In contrast, of the 46 patients without malignancy, 34 tested positive for gDNA. For plasma-based ctDNA, 27 patients were positive, 60 were negative, and among the 27 positive patients, 24 were also positive in gDNA. Among the 60 negative patients, 50 were positive in gDNA.

The vaginal swab-based gDNA results were used to calculate the microsatellite instability (MSI) score (Fig. 1). This score was computed based on 50 MSI target sites, counting each site with an nBi score of 1 or higher. The total count of sites was multiplied by 2, resulting in a score of 100. MSI evaluation included patients tested for 2 or more of *MSH2*, *MSH6*, *PMS2*, and *MLH1*, with those losing at least one gene considered MSI detected. Comparing vaginal swab-based gDNA NGS-based MSI scores with tissue MSI detection, a cutoff above 10 showed a sensitivity of 0.67, specificity of 0.86, and accuracy of 0.81, indicating high performance and designating this cutoff as MSI High (MSI-H). Setting the cutoff above 20 resulted in a sensitivity of 0.42, specificity of 1.00, and accuracy of 0.86, making it a second choice for MSI-H. With a cutoff of 10, Cohen's Kappa was 0.501, indicating moderate agreement.

A coplot was generated for patients with positive results from vaginal swab-based gDNA (Fig. 2), analyzing tier I, II, and III, and tiers I and II. In both analyses, *PTEN*, *PIK3CA*, *ARID1A*, *TP53*, *KRAS*, and *CTNNB1* ranked high.

The ProMisE classification was applied based on mutations identified in endometrial cancer patients (Fig. 3). The MMR-D group was calculated for an MSI score above 10, comprising 25 patients (28.7%). The *POLE* EDM group included 7 patients (8.0%) with tier I or II mutations in the *POLE* gene. For *TP53*, 14 patients (16.1%) had tier I or II mutations, while 8 patients (9.2%) with *CTNNB1* had tier I or II mutations, and 33 patients (37.9%) were *CTNNB1* normal. Comparisons with the original ProMisE classification showed differences of less than 5 percentage points across all groups. Kaplan–Meier curves for the ProMisE-defined groups indicated particularly low disease-free survival rates for the p53 abn group (Fig. 4), consistent with previous literature.

Among the 87 patients with positive vaginal swab-based gDNA results, this study used the ProMisE classification based on IHC results to assess prognosis. A total of 66 patients were eligible for classification, as most had undergone IHC testing for MMR genes (*MSH2*, *MSH6*, *PMS2*, *MLH1*) and p53. However, routine testing for *POLE* IHC was not performed, and only a few patients had undergone this test. Therefore, patients not classified as MMR-D but had positive *POLE* IHC results or tier I or II mutations identified by NGS were categorized into the *POLE* EDM group.

Based on these criteria, of the 66 patients, 14 (21.2%) were classified as MMR-D, 8 (12.1%) as *POLE* EDM, 10 (15.2%) as p53 abn, and 34 (51.5%) as p53 wt. Recurrence rates among these groups were as follows: 1 patient (7.1%) in the MMR-D group, 1 patient (12.5%) in the *POLE* EDM group, 3 patients (30.0%) in the p53 abn group, and 6 patients (17.6%) in the p53 wt group, with the highest recurrence rate in the p53 abn group.

Table 5. Demographic features of the vaginal swab-based gDNA samples

Characteristic		*n*
Age (years)	Mean (SD)	53.3(13.1)
Stage	Benign	17
	AH/EIN	12
	IA	63
	IB	18
	II	14
	III	11
	IV	6
Pathology	Adenosarcoma	1
	Carcinosarcoma	7
	Clear cell	5
	Dedifferentiated	1
	Endometroid	96
	Serous	1
	Stromal Sarcoma	1
Vaginal swab-based gDNA	Negative	45
	Borderline	8
	Positive	88

Table 6. Results of the vaginal swab-based gDNA samples

		Vaginal swab-based gDNA			Total	*p*-value
		Negative	Borderline	Positive		
Stage	Benign	14	2	1	17	<0.01
	AH/EIN	10	2	0	12	(AH/EIN as benign)
	IA	18	3	42	63	
	IB	1	0	17	18	(IB~IV as "Not IA")
	II	1	0	13	14	
	III	1	0	10	11	
	IV	0	1	5	6	
Cytology	Negative	13	1	12	26	0.03
	Atypical/LSIL	7	1	22	30	(Borderline as Negative)
	Malignancy	1	0	8	9	
Lymphovascular invasion	No	18	2	54	74	0.06
	Yes	2	1	32	35	
Plasma based-ctDNA	Negative	12	2	35	49	0.46
	Borderline	5	1	15	21	
	Positive	2	1	24	27	
Prognosis	NED	49	3	69	121	0.01
	Recur/death	1	1	16	18	(Except F/U loss)
	F/U Loss	0	0	2	2	

Table 7. Comparison of vaginal swab-based gDNA, PAP cytology, and plasma-based ctDNA in patients with endometrial cancer

		Vaginal Swab-based gDNA	
		Negative	Positive
PAP cytology	Negative	12	34
	Positive	1	9
Plasma-based ctDNA	Negative	10	50
	Positive	3	24

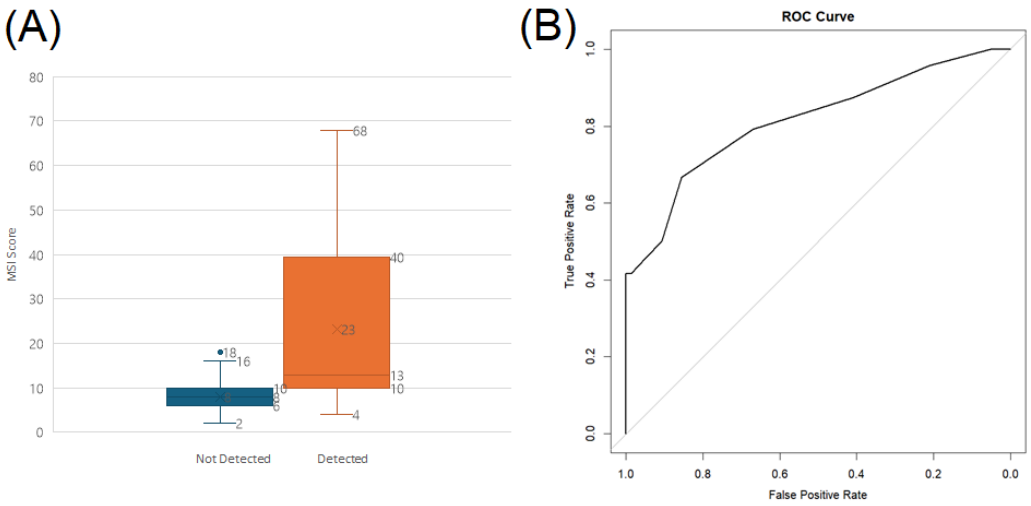


Figure 1. MSI score assessment using NGS. **A** Box plot shows the distribution of MSI scores calculated by NGS for patients classified as MMR-detected and MMR-not detected. **B** ROC curve illustrates the performance of the NGS-based MSI scores in predicting the MMR status.

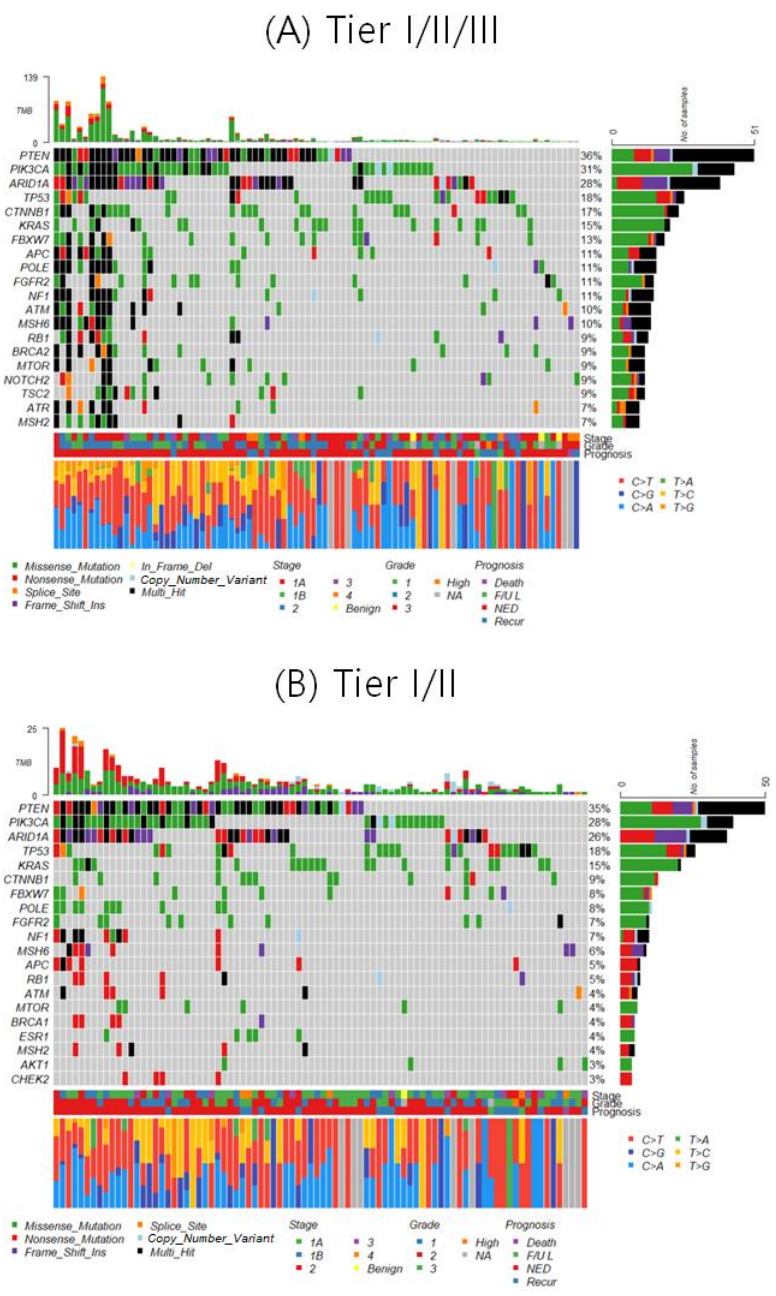


Figure 2. Oncoplots of vaginal swab-based gDNA samples. **A** Mutations of tier I, II, and III. **B** Mutations of tier I and II

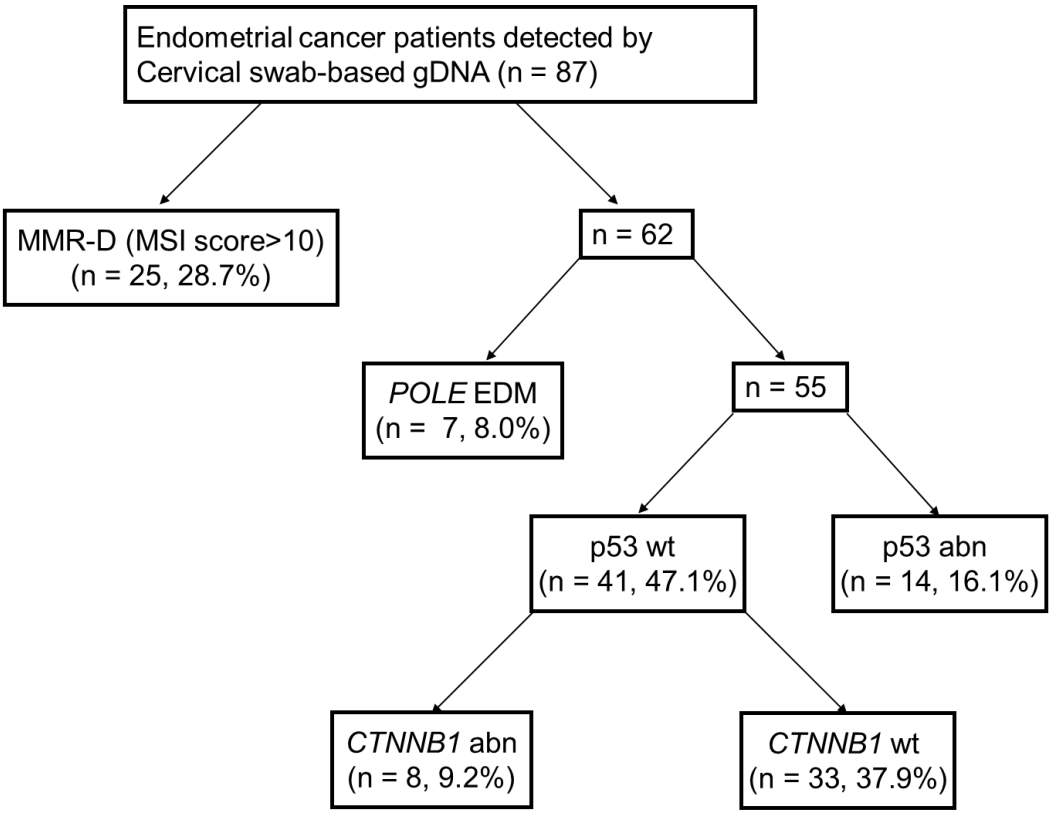


Figure 3. ProMisE classification of vaginal swab-based gDNA samples

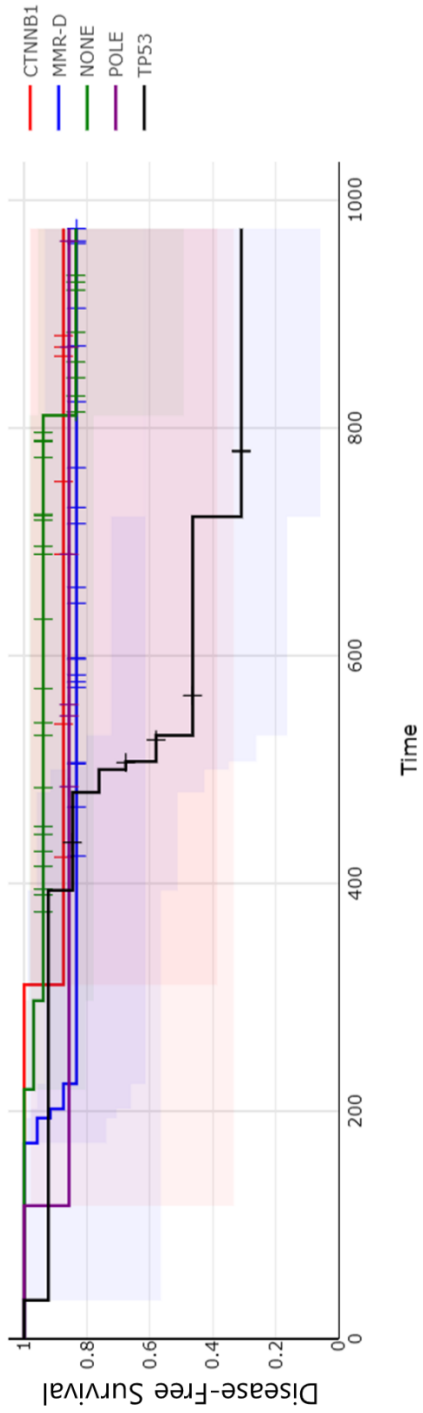


Figure 4. Kaplan–Meier curve for the ProMisE classification of vaginal swab-based gDNA samples

Among the endometrial cancer patients with positive vaginal swab-based gDNA results, Cox regression was performed for both the univariate and the multivariate analyses (Table 8). In the univariate analysis, advanced stage, lymphovascular invasion, *PTEN* mutation, and *TP53* mutation were significantly associated with disease-free survival. The multivariate analysis confirmed that lymphovascular invasion and *PTEN* mutation were significantly associated with disease-free survival. *PTEN* mutation positively influenced prognosis in both analyses.

To evaluate the significance of *PTEN* and *TP53*, this study referenced the Cancer Genome Atlas database and analyzed the TCGA-UCEC data(9), which showed that both *PTEN* and *TP53* were significantly linked to survival (Fig. 5).

Based on the ProMisE classification, Cox regression, and the Cancer Genome Atlas database, the *PTEN* mutation was found to have a positive impact on prognosis, while the *TP53* mutation had an adverse impact. Patients were categorized into groups of both mutated, *PTEN* mutated, *TP53* mutated, and neither mutated, and Kaplan–Meier curves were plotted (Fig. 6A). Kaplan–Meier curves were plotted, revealing nearly identical prognoses for both mutated and *PTEN* mutated groups. Additional curves were created for patients with and without *PTEN* mutations (Fig. 6B), as well as for those with and without *TP53* mutations (Fig. 6C). All three Kaplan–Meier curves had *p*-values below 0.05 (Fig. 6A: <0.01; Fig. 6B: 0.01; Fig. 6C: <0.01).

A new approach divided patients into *PTEN*-mutated and *TP53*-mutated groups (Fig. 7A). The 50 patients in the *PTEN* mutated group were considered to have good to moderate prognosis. Among the remaining 35 patients, those with *TP53* intact were categorized as having a moderate prognosis, and those with *TP53* mutations were categorized as having a worse prognosis. Kaplan–Meier curves related to their prognosis were also presented (Fig. 7B).

Mutation plots were created for genes with high mutation frequencies (*TP53*, *PIK3CA*, *ARID1A*, *PTEN*, *KRAS*, *CTNNB1*) (Fig. 8). *PIK3CA*, *KRAS*, and *CTNNB1* showed mutations concentrated in hotspots, while *TP53*, *ARID1A*, and *PTEN* had a more scattered mutation pattern.

Table 8. Univariate and multivariate Cox regression analysis for vaginal swab-based gDNA-positive endometrial cancer patients

Cox regression univariant				
		HR	95% CI	*p*-value
Stage	Advanced	7.1	2.6–19	0.00011*
Age	≥65	1.4	0.49–4.1	0.53
Lymphovascular invasion	Present	9.4	2.7–33	0.00049*
Gene	*PTEN*	0.27	0.093–0.78	0.016*
	Multi-*PTEN*	0.84	0.27–2.6	0.76
	PIK3CA	0.36	0.12–1.1	0.08
	ARID1A	0.4	0.13–1.2	0.11
	TP53	3.6	1.3–9.8	0.011*
	KRAS	1.3	0.42–4.1	0.63
	CTNNB1	0.78	0.18–3.4	0.74
	FBXW7	1.7	0.48–5.9	0.41
	POLE	0.44	0.058–3.3	0.42
	FGFR2	0.47	0.062–3.6	0.47
	NF1	0.47	0.062–3.6	0.47
	MSH6	1.3	0.3–5.8	0.72
	APC	1.8	0.8.1	0.42
	RB1	1.1	0.14–8.3	0.94
Cox regression multivariate				
			Exp(coef))	Pr(>lzl)
Stage	Advanced		1.3598	0.63818
Lymphovascular invasion	Present		9.167	0.00342*
Gene	*PTEN*		0.239	0.01852*
	TP53		2.2038	0.14423

*p-value<0.05

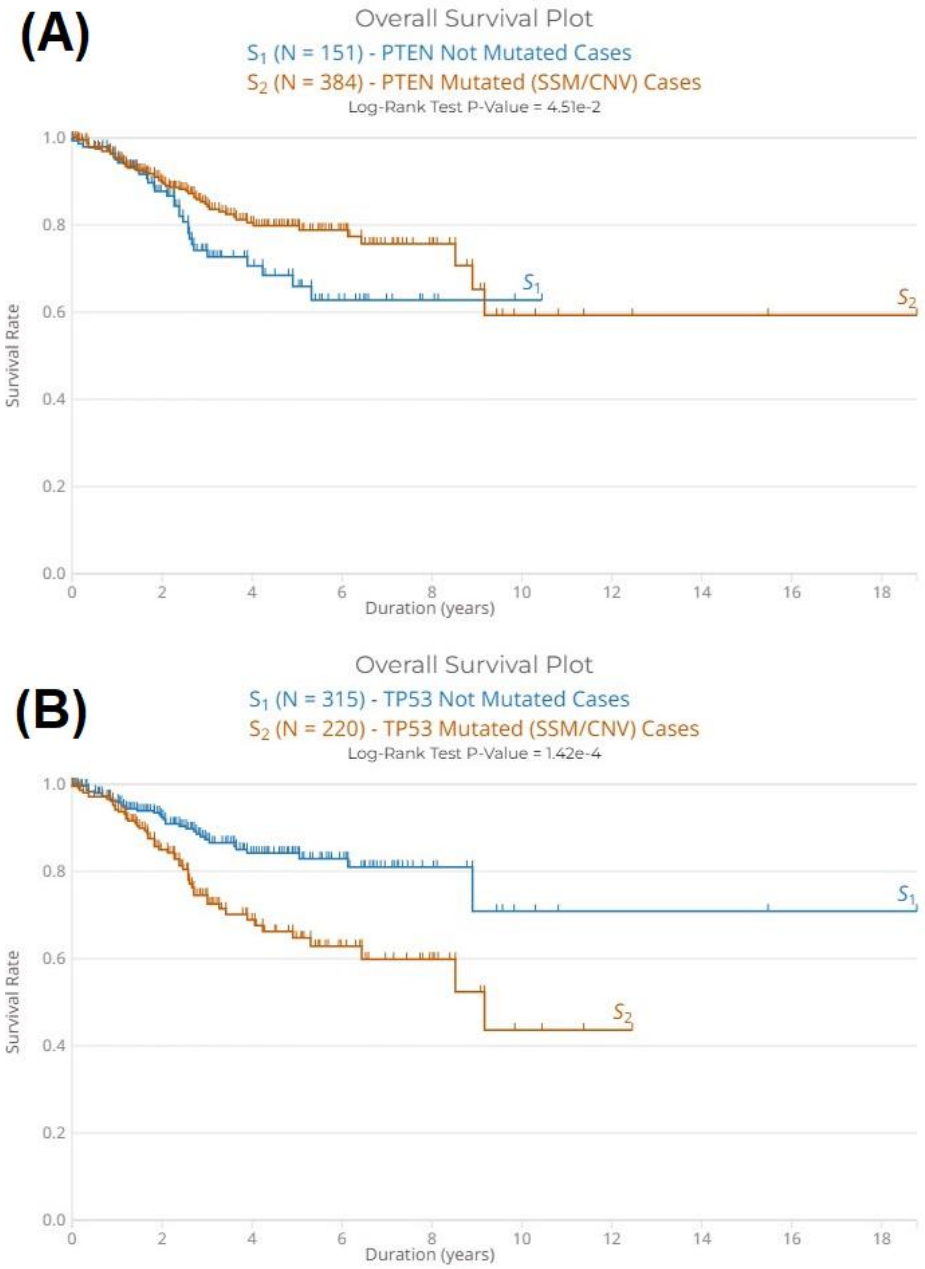


Figure 5. Kaplan–Meier survival curves in endometrial cancer using TCGA-UCEC data. **A** *PTEN*, **B** *TP53*

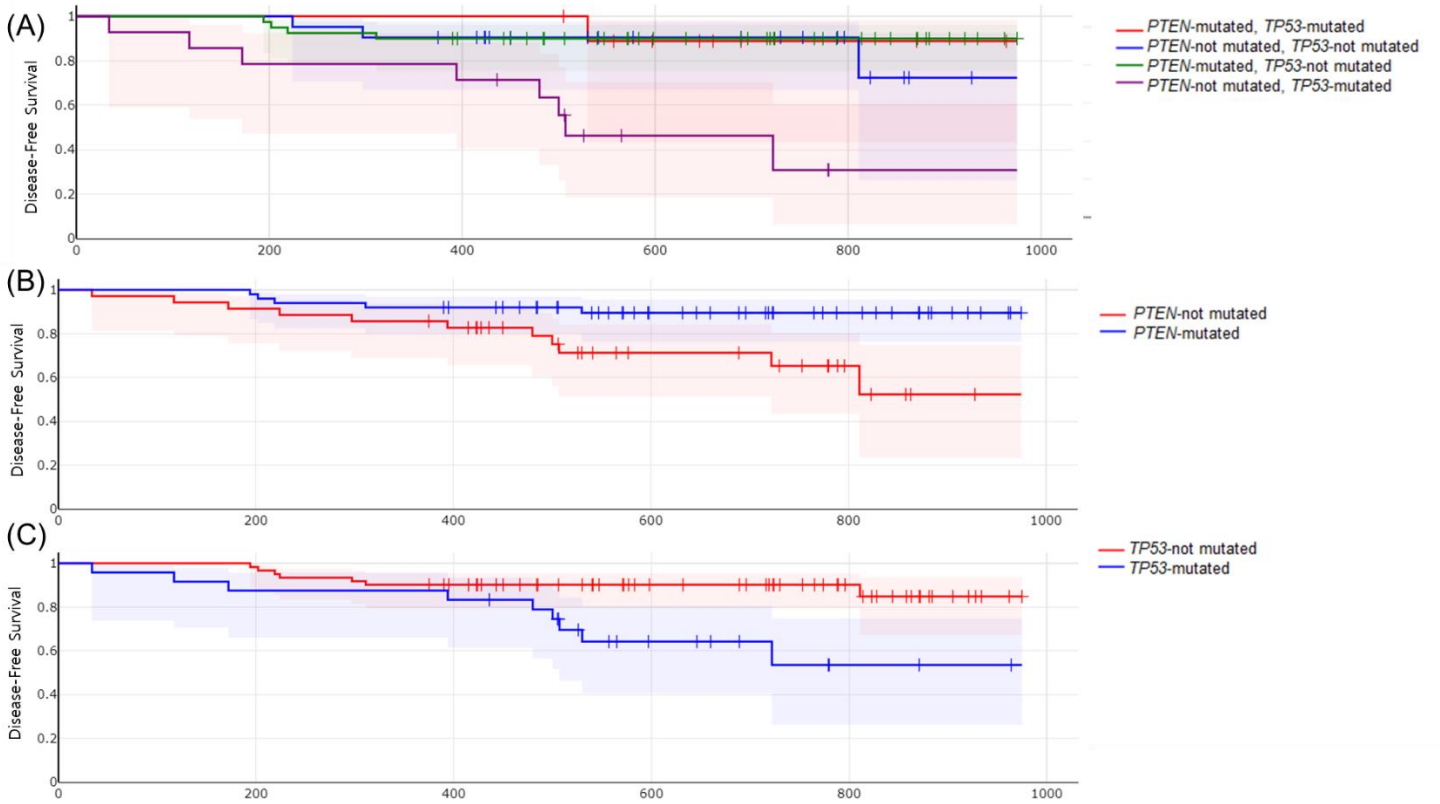


Figure 6. Kaplan–Meier survival curves by *PTEN* and *TP53* status. **A** Kaplan–Meier survival curves for combinations of *PTEN* and *TP53* statuses. **B** Kaplan–Meier survival curves for patients based on *PTEN* status alone. **C** Kaplan−Meier survival curves for patients based on *TP53* status alone

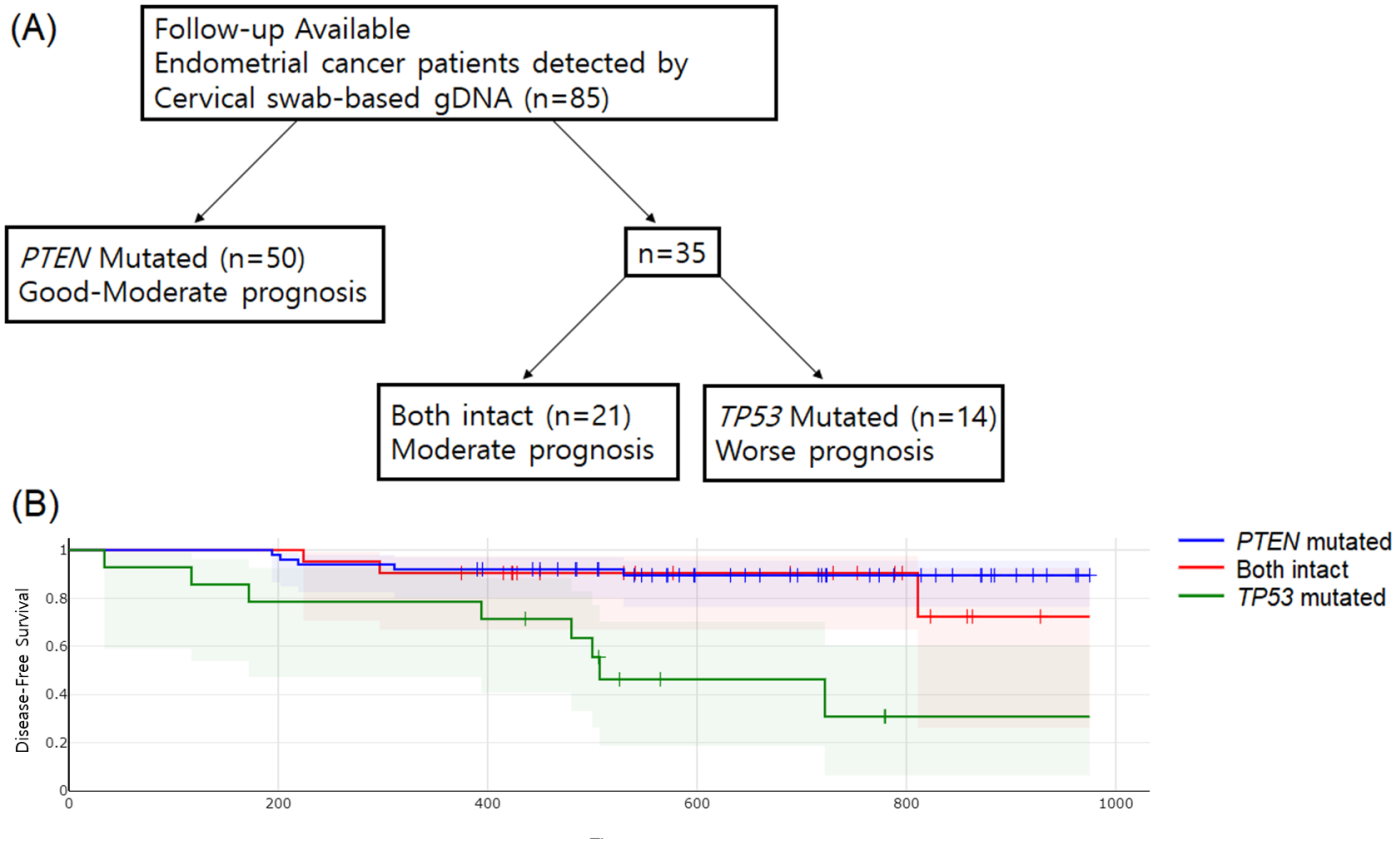


Figure 7. New classification approach for endometrial cancer based on *PTEN* and *TP53* status. **A** Classification schema. **B** Kaplan–Meier survival curves

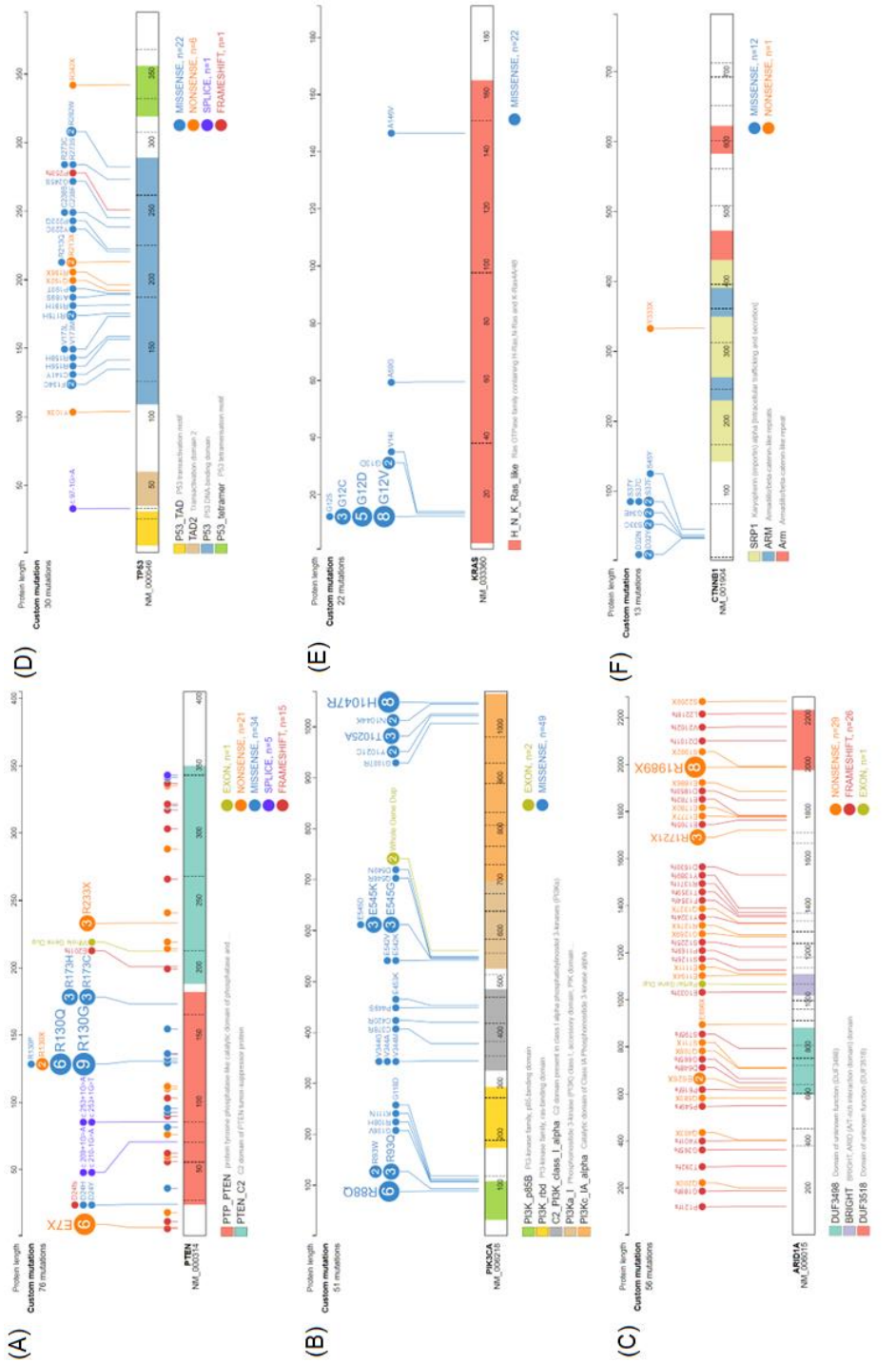


Figure 8: Mutation plot (7-1. *PTEN*, 7-2. *PIK3CA*, 7-3. *ARID1A*, 7-4. *TP53*, 7-5. *KRAS*, 7-6. *CTNNB1*) for tiers I and II mutations.

3.3. Analysis of Plasma-based ctDNA

This section discusses the plasma-based ctDNA analysis results for 22 patients with benign or AH/EIN and 123 patients with endometrial cancer. Blood samples were collected once for benign and AH/EIN patients, while endometrial cancer patients had up to 6 blood collections.

Table 9 shows the demographics for 145 endometrial cancer patients, with an average age of 54.8 years. Among the 123 endometrial cancer patients, there were 50 at stage IA, 25 at advanced stages (stages III and IV), and 18 with recurrence or rerecurrence. Pathology analysis revealed that endometrioid cancer was the most common, with 89 cases.

Plasma-based ctDNA was separately analyzed for stage, lymphovascular invasion, and prognosis (Table 10). Among the 123 endometrial cancer patients, 31 (25.2%) showed positive results, with 24 (42.9%) in stages IB to IV and 9 (36%) in advanced stages (stages III, IV). There was a significant difference in positivity rates, with 14 out of 66 patients (21.2%) without lymphovascular invasion testing positive compared to 16 out of 39 (41.0%) with lymphovascular invasion. Prognostically, the recurrence/death group exhibited significantly higher ctDNA positivity.

Kaplan–Meier curves were plotted to compare disease-free survival between patients with positive ctDNA results and those with negative or borderline results (Fig. 9). The analysis showed a significant difference in disease-free survival ($p < 0.01$).

For primary endometrial cancer patients without recurrence, Cox regression univariate analysis was conducted to assess prognosis based on ctDNA positivity, age (65 years and older), advanced stage (stage III or IV), and lymphovascular invasion (Table 11). The analysis identified ctDNA positivity, advanced stage, and lymphovascular invasion as significant factors. A subsequent Cox multivariate analysis confirmed that lymphovascular invasion was a significant predictor of prognosis.

Oncoplots were generated based on tier I, II, and III mutations and tier I and II mutations separately using plasma-based ctDNA (Fig. 10). *TP53*, *PTEN*, *ARID1A*, and *PIK3CA* genes were prominent, showing some variation compared to rankings based on vaginal swab-based gDNA.

The most notable variation was observed in *TP53*. Due to reports of high rates of clonal hematopoiesis of indeterminate potential in *TP53*, additional NGS was performed using peripheral blood mononuclear cells (PBMC) for cases where no mutation was detected in tissue NGS or vaginal swab-based gDNA. Clonal hematopoiesis was confirmed in one case, and this mutation was excluded from the overall report. The remaining 16 patients had high rates of advanced stage or recurrence (Fig. 11).

Table 9. Demographic features of the plasma-based ctDNA samples

Age	*m*, SD	54.8(13.1)
Stage	Benign	13
	AH/EIN	9
	IA	50
	IB	19
	II	12
	III	12
	IV	13
	Recur	9
	Rerecur	9
Pathology	Adenosarcoma	1
	Carcinosarcoma	7
	Clear cell	4
	Dedifferentiated	1
	Endometroid	89
	Serous	3
	Stromal sarcoma	1
1st Plasma-based ctDNA result	Negative	72
	Borderline	33
	Positive	41
Number of ctDNA tests performed	1	102
	2	12
	3	17
	4	8
	5	5
	6	2

Table 10. Results of the first plasma-based ctDNA samples

		Plasma-based ctDNA results				
		Negative	Borderline	Positive	Total	*p*-value
Stage	Benign	10	2	1	13	<0.01
	AH/EIN	7	1	1	9	(IB~IV as 'Not IA')
	IA	31	14	5	50	
	IB	12	3	4	19	
	II	4	2	6	12	
	III	0	4	8	12	
	IV	2	2	9	13	
	Recur	3	3	3	9	
	Rerecur	3	2	4	9	
Lymphovascular invasion (Only cancer)	No	38	16	11	65	<0.01
	Yes	11	8	20	39	
Prognosis	NED	66	24	19	109	<0.01
	Recur/death	5	8	21	34	(Except F/U loss)
	F/U Loss	1	1	1	3	

Table 11. Univariate and multivariate Cox regression analysis for plasma-based ctDNA in patients with endometrial cancer

Cox regression univariant				
		HR	95% CI	*p*-value
Stage	Advanced	5.7	2.3–14	<0.01**
Age	≥65	1.6	0.63–4.2	0.32
Lymphovascular invasion	Present	11	3.3–38	<0.01**
Plasma-based ctDNA	Positive	6.1	2.4–15	<0.01**
Cox regression multivariate				
			Exp(coef))	Pr(>lzl)
Stage	Advanced		1.5736	0.39199
Lymphovascular invasion	Present		6.8649	<0.01**
Plasma-based ctDNA	Positive		3.0825	0.03*

*p-value <0.05, **p-value <0.01

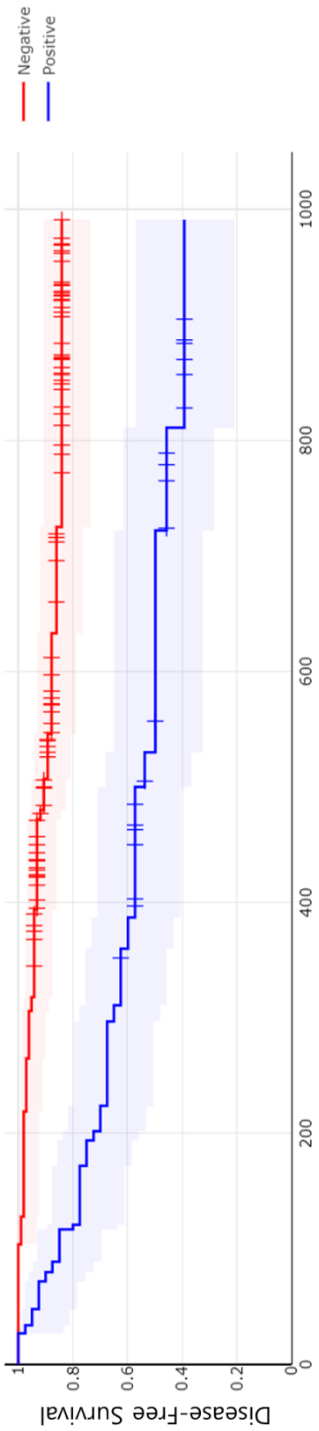


Figure 9. Kaplan–Meier curve for plasma-based ctDNA result in patients with endometrial cancer

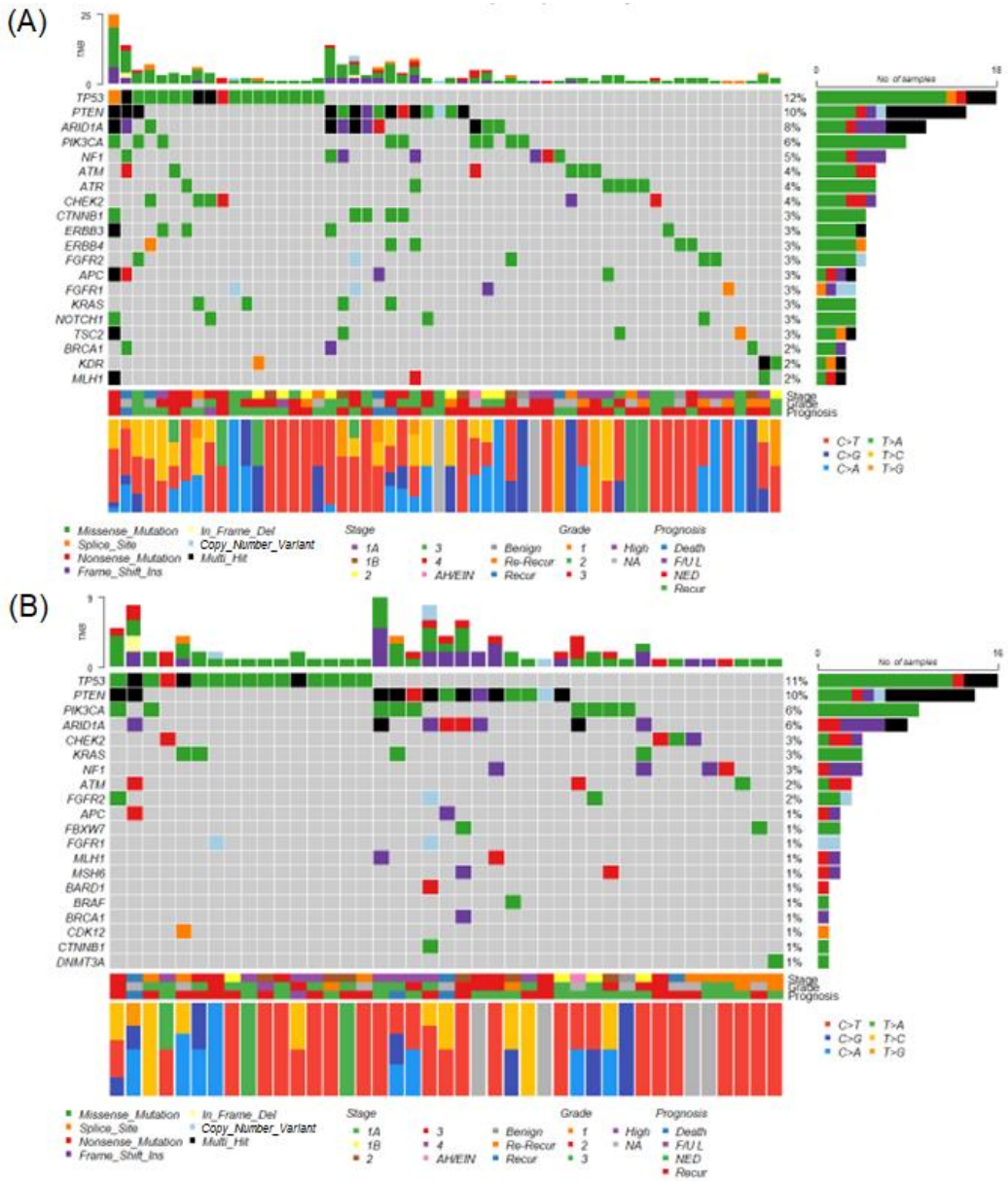


Figure 10. Oncoplots of plasma-based ctDNA samples. **A** Mutations of tier I, II, and III. **B** Mutations of tier I and II

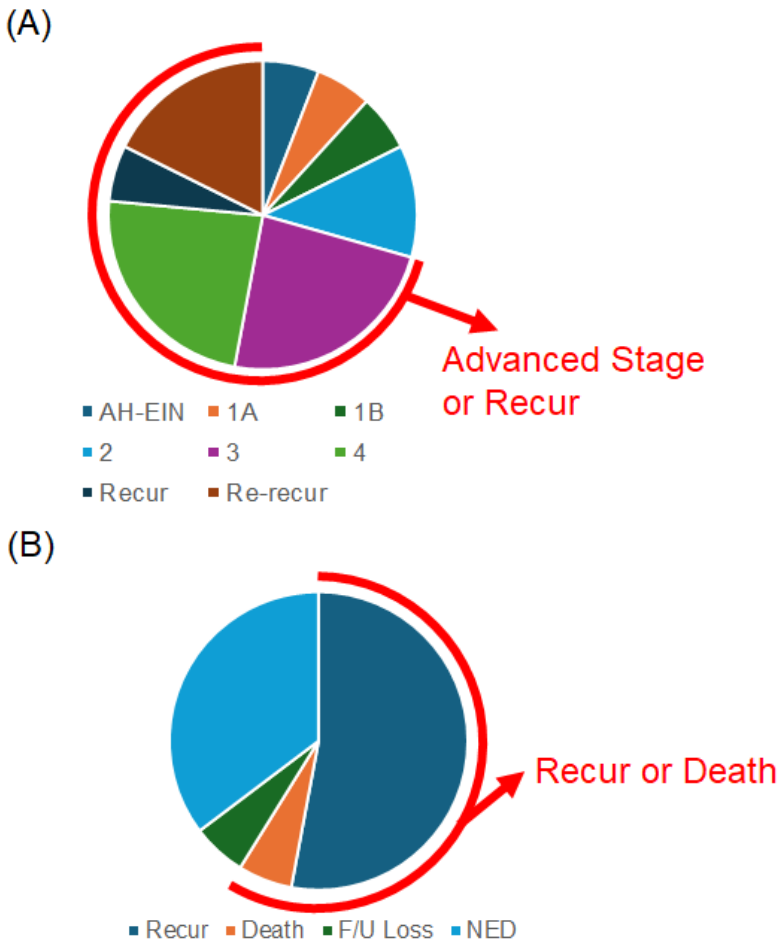


Figure 11. Analysis of the *TP53*-positive patient group based on plasma-based ctDNA. **A** Stage analysis. **B** Prognostic analysis

Analysis was conducted on the initial plasma-based ctDNA data and on 44 patients who underwent two or more plasma-based ctDNA sampling. Many of these patients were classified as stage I, and a significant proportion showed NED regarding prognosis. Therefore, the analysis concentrated on those with multiple plasma-based ctDNA samplings, particularly those who experienced a recurrence.

A Sankey plot was generated for patients who underwent plasma-based ctDNA testing two or three times (Fig. 12). Most patients with negative plasma-based ctDNA results prior to surgery remained negative postoperatively. Additionally, many patients who initially tested positive became negative within 2–4 months following surgery. Only a few patients had positive plasma-based ctDNA results at the 2–4 month mark. However, patients tested for plasma-based ctDNA both 2–4 months and 5–7 months after surgery displayed a different trend: a certain percentage of patients from the 2–4 month postoperative group tested positive again, resulting in a higher positivity rate in the 5–7 month postoperative group compared to the 2–4 month group.

An analysis was conducted on 16 patients who experienced recurrence among those with two or more plasma-based ctDNA samples, resulting in the creation of a Swimmer plot (Fig. 13). In patients with regular plasma-based ctDNA collections, a correlation was observed between recurrence and positive ctDNA results. However, no significant results were observed in cases with extended sampling intervals or inconsistent follow-up.

Figure 14 summarizes the changes in the variant allele frequency (VAF) for cases where recurrence was successfully predicted using plasma-based ctDNA. In this study, the VAF cutoff for plasma-based ctDNA was set at 0.05%. The average depth of vaginal swab-based gDNA was over 35,000×, while plasma-based ctDNA exceeded 40,000×. This high sequencing depth allowed for an effective VAF cutoff of 0.05%, enabling accurate detection of recurrence.

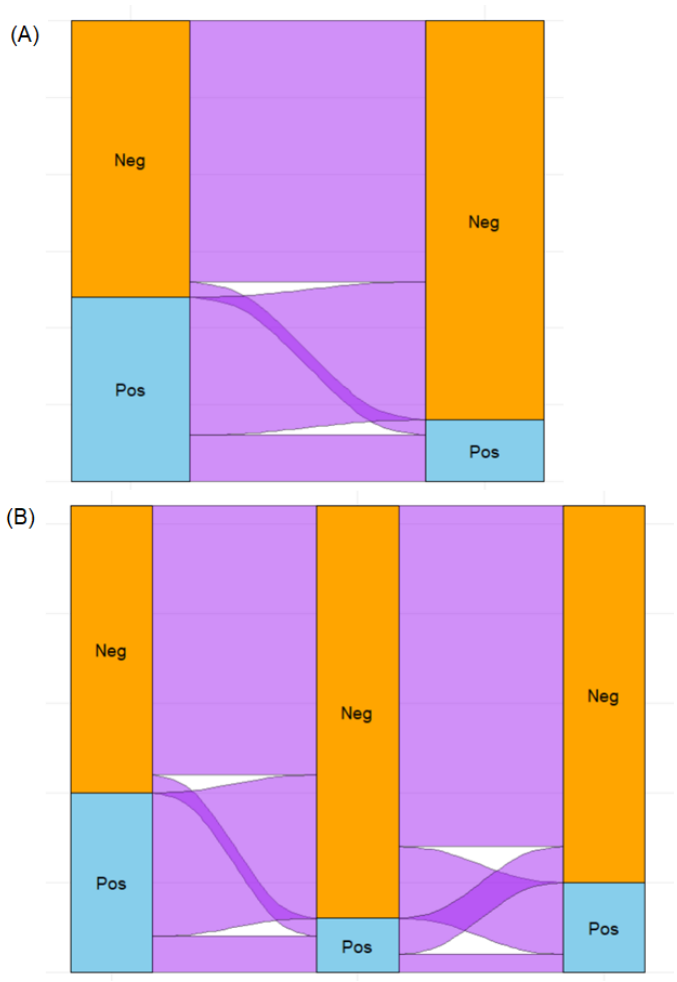


Figure 12. Sankey plots for plasma-based ctDNA. **A** Comparison of plasma-based ctDNA results between presurgery and 2–4 months postsurgery. **B** Sequential comparison of the plasma-based ctDNA results across three time points, 2–4 months postsurgery and 5–7 months postsurgery.

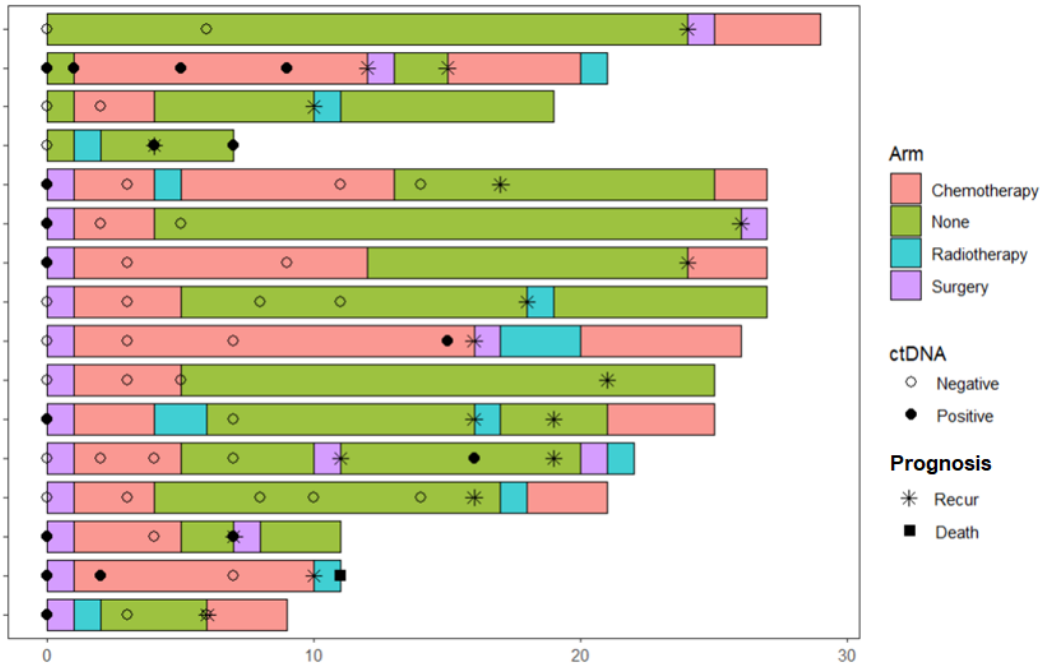


Figure 13. Swimmer plot illustrating the treatment arms, including chemotherapy, radiotherapy, and surgery, alongside plasma-based ctDNA status and patient outcomes.

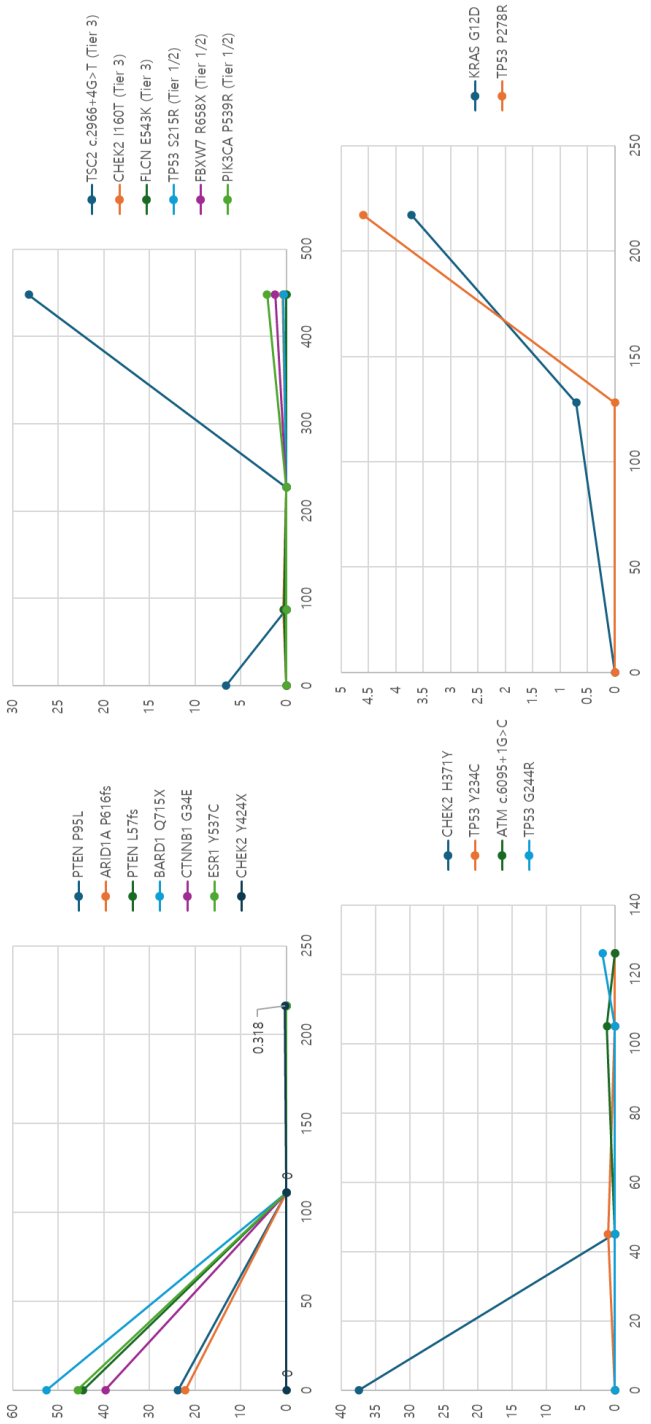


Figure 14. Variants of allele frequency comparison for four patients in whom plasma-based ctDNA successfully predicted recurrence

3.4. Comparison with Tissue-based NGS

One objective of this study was to determine whether vaginal swab-based gDNA could assist in diagnosis. An analysis was conducted on patients who underwent tissue-based NGS. Out of 26 patients who underwent tissue-based NGS, 12 had recurrence or rerecurrence and subsequently underwent plasma-based ctDNA analysis (Table 12). Consequently, direct comparisons with tissue NGS were limited to the 14 patients with first-time diagnosed endometrial cancer.

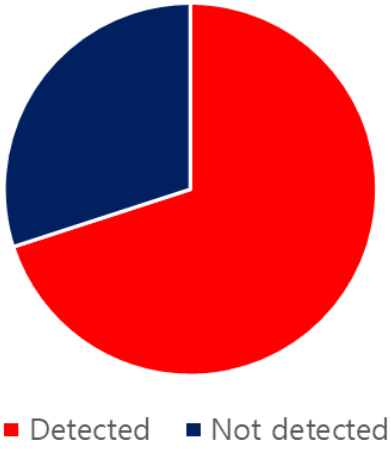
Among the 14 patients, significant mutations were observed in 13, including 7 with *TP53* mutations and 5 with *PTEN* mutations, aligning with the focus of this study on these genes. The frequently mutated genes in the vaginal swab-based gDNA and plasma-based ctDNA analyses were similar; *PIK3CA*, *ARID1A*, *CTNNB1*, and *KRAS* mutations were found in 5 patients each. Notably, 9 out of the 14 patients (64%) were classified as advanced stage (stage III or IV), which is unusual given the high early-stage statistics typically seen in endometrial cancer. This could be due to the common practice of prescribing tissue NGS primarily for advanced-stage cases, explaining the high proportion of *TP53* mutations. Similarly, *TP53* was identified as the most frequently mutated gene in plasma-based ctDNA, likely due to its association with poorer prognoses, as 10 out of the 14 patients experienced recurrence.

An analysis was conducted to determine whether the same mutations were detected (Fig. 15). Among the 10 patients who underwent both tissue-based NGS and vaginal swab-based gDNA analyses, 7 exhibited identical mutations. Similarly, among the 14 patients who underwent both tissue-based NGS and plasma-based ctDNA analyses, 7 also demonstrated the same mutations, while 2 showed discrepancies despite sharing the same mutations. In the two patients, discrepancies were observed between tissue-based NGS and plasma-based ctDNA. In one patient, tissue-based NGS identified mutations including *AKT1* p. E17K, *CTNNB1* p. G34R, *PHF6* p. K21*, *BCOR* p. B1459S, and *ZFHX3* p. Q1792*, whereas plasma-based ctDNA revealed a *PTEN* partial gene deletion. In the other patient, no mutations were detected by tissue-based NGS, but plasma-based ctDNA identified a *TP53* p. Y234N mutation with a VAF of 6.45%.

Table 12. Demographic features and results of tissue-based NGS in 14 patients with newly diagnosed endometrial cancer

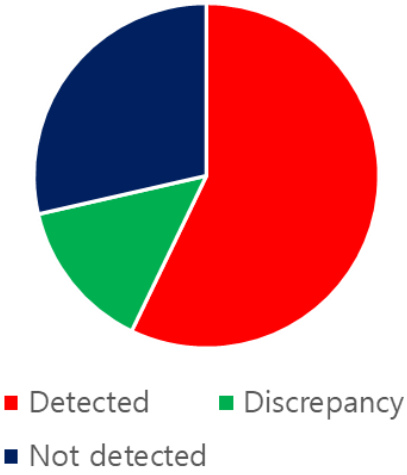
Characteristic		*n* (%)
Result	Mutation detected	13 (93%)
	No mutation detected	1 (7%)
Stage	I, II	5 (36%)
	III, IV	9 (64%)
Prognosis	Recur	10 (71%)
	No evidence of the disease	4 (29%)
Genes	*TP53*	7 (50%)
	PTEN	5 (36%)
	PIK3CA	5 (36%)
	ARID1A	5 (36%)
	CTNNB1	5 (36%)

(A)



Detected gene	n
TP53	4
PTEN	1
ARID1A	1
KRAS	1
AKT1	1
CTNNB1	1

(B)



Detected gene	n
TP53	5
PTEN	3
PIK3CA	2
KRAS	2
ESR1	1
CTNNB1	1
BARD1	1
ARID1A	1

Figure 15. Comparison of tumor-based NGS results with **A** vaginal swab-based gDNA and **B** plasma-based ctDNA in patients.

IV. Discussion

In this study, this study thoroughly analyzed vaginal swab-based gDNA and plasma-based ctDNA in patients with endometrial cancer, comparing these results with other diagnostic methods. Main objectives were to determine if vaginal swab-based gDNA could aid in genomic profiling for endometrial cancer, explore its potential for identifying prognostic factors, and assess plasma-based ctDNA monitoring for effective patient follow-up. This findings demonstrated several positive outcomes in line with these objectives, emphasizing that not only the binary results of NGS testing (positive or negative) but also the specific genetic mutations detected are critical for clinical interpretation.

This study prospectively collected samples from 191 patients, with 42 benign and 149 cancer cases, analyzing a total of 386 samples. The number of cancer cases was significantly higher than that in pilot study, which included 39 cancer patients and 11 benign cases. While the pilot study had already achieved a high sequencing depth, its VAF cutoff was not low enough(8). In contrast, this study reanalyzed the pilot samples with a lower VAF cutoff to match the high sequencing depth. This study also expanded the analysis to include tier III variants as well as assessments of MSI and prognostic factors, representing substantial improvements over the pilot study. Unlike previous studies that analyzed 101 genes using the PiSeq algorithm(25), this study also included plasma-based ctDNA alongside vaginal swab-based gDNA analysis. Lowering the VAF cutoff enhances sensitivity but can decrease specificity, which presents a trade-off. This study achieved 77.7% sensitivity and 96.6% specificity, compared to the pilot study's sensitivity of 67% and specificity of 100%(8). While specificity slightly decreased due to one case, sensitivity improved by over 10 percentage points, indicating that deep sequencing in this study allowed for mutation detection that the pilot study missed.

For instance, one patient diagnosed with a benign condition showed positive results in vaginal swab-based gDNA, revealing mutations in *KRAS* p. G12V and *PIK3CA* p. H1047R, which are common in endometrial cancer(26). The patient had previously been suspected of having endometrial cancer 3 years prior after a dilation and curettage procedure, which resulted in a stage IA diagnosis. Although the subsequent hysterectomy found no malignancy, the presence of these genetic alterations could indicate clonal hematopoiesis of indeterminate potential, similar to what occurs in hematologic disorders(27). It is plausible that genetic mutations were present in a small population of cells within the reproductive system, contributing to the malignancy 3 years ago but remained benign in the current study. However, the limited number of similar cases in this cohort restricts further interpretation of this phenomenon.

In both the pilot study and the current analysis, comparisons with PAP smears were also conducted(8). Among the 46 endometrial cancer patients with negative PAP results, 34 tested positive using vaginal swab-based gDNA, highlighting its superior sensitivity. However, of the 10

patients with adenocarcinoma detected by PAP cytology, one yielded a negative result in the vaginal swab-based gDNA test. This patient had a clear cell carcinoma, stage IA, detected by PAP smear. In this study, 3 of the 5 patients with clear cell carcinoma had detectable mutations using vaginal swab-based gDNA, suggesting the negative result was likely due to insufficient tumor sampling during the swab procedure. Among the 24 patients who underwent tissue-based NGS, 14 were newly diagnosed with endometrial cancer, and 1 had no detectable mutations, indicating that vaginal swab-based gDNA testing may not always yield definitive results. Nevertheless, as in the pilot study, vaginal swab-based gDNA outperformed PAP smear as a noninvasive diagnostic method.

The pilot study successfully used the ProMisE classification to define the MMR-D group based on mutations in *MLH1*, *PMS2*, *MSH2*, and *MSH6*(8, 12). This study explored alternative approaches that focus on tumor mutation burden (TMB), MSI, and MMR due to changing research trends(28, 29). Instead of solely relying on the MMR approach, this study targeted 50 well-known MSI sites, including *MSH2*, *MSH6*, *ATM*, and *RAD51B*(30). This study validated MSI scores against MMR measured by immunohistochemistry (IHC), yielding statistically significant results. However, the concordance between NGS-based MSI and IHC-based MMR in endometrial cancer was not particularly high, showing a sensitivity of 55% and specificity of 97%(31). At a cutoff of 10, sensitivity was 0.67, specificity was 0.86, and accuracy was 0.81. At a cutoff of 20, sensitivity was 0.42, specificity was 1.00, and accuracy was 0.86. Although the accuracy was higher at a cutoff of 20, the significant sensitivity at a cutoff of 10 suggested it was more logical for assessing MMR-d in endometrial cancer, with 20 as a secondary option for future research.

Given this updated approach to MMR through MSI, this study also reevaluated the ProMisE classification(12). While this classification has been groundbreaking, it has some limitations. In particular, the *POLE* EDM group did not show significant correlations with overall survival, disease-specific survival, or progression-free survival. The MMR-D group showed modest results, with the highest hazard ratio being 2.310 and only borderline significance for progression-free survival. The most notable finding across all survival metrics was the poor prognosis associated with the p53 abnormal group.

Despite these limitations, the *POLE* EDM group showed better prognoses than the p53 mutant group, highlighting the prevalence of low-risk patients within the *POLE* EDM group. Multiple studies supporting the ProMisE classification consistently identify the *POLE* EDM group as having the best overall prognosis(32, 33). Although some studies have failed to show a statistically significant difference in prognosis between the *POLE* EDM and p53 wild-type groups, many relied on IHC methods(34). Considering the available genetic markers (MMR genes, p53, *POLE*), the ProMisE classification remains the best possible approach using IHC.

Analyzing vaginal swab-based gDNA remains more expensive than testing all four MSI genes, *POLE*, and p53 through IHC methods. This highlights the need to optimize gene selection

and adjust sequencing depth for better cost-effectiveness. However, because this study already performed deep sequencing of 101 genes, this study conducted a Cox regression analysis of all candidate genes and identified *PTEN* as a significant prognostic factor.

This study referenced the Cancer Genome Atlas database and conducted an analysis using the TCGA-UCEC data(9), demonstrating that mutations in *PTEN* or *TP53* influenced prognosis. Another study using databases from cBioPortal also identified *PTEN* as a favorable prognostic biomarker in endometrial cancer(35), while *TP53* has been consistently associated with poor prognosis, supporting the ProMisE classification(32-34).

This study classified patients based on *PTEN* and *TP53* mutations. The *PTEN*-mutant group and the group with intact *TP53* and *PTEN* showed similar prognoses, with the *PTEN*-mutant group displaying a slight advantage. Importantly, patients with simultaneous *PTEN* and *TP53* mutations had significantly better outcomes than those with *TP53* mutations alone, aligning more closely with the *PTEN*-only mutant group. Although *PTEN* mutations are typically associated with poor prognosis in other cancers(36, 37), studies in colorectal cancer found improved survival with *PTEN* mutations in MSI-positive tumors(38). Similarly, early reports from 1998 and more recent studies have linked *PTEN* mutations in endometrial cancer with favorable outcomes, particularly in early-stage, nonmetastatic disease(13, 39). However, the occurrence of simultaneous *PTEN* and *TP53* mutations is rarely discussed(40), highlighting the need for further research.

A mutation plot was generated for the six most frequently mutated genes found in vaginal swab-based gDNA (*TP53*, *PIK3CA*, *ARID1A*, *PTEN*, *KRAS*, and *CTNNB1*). The analysis revealed that the majority of the mutations in *PIK3CA*, *KRAS*, and *CTNNB1* were in known hotspot regions. All missense mutations in *PIK3CA* occurred within the hotspots, while all but two mutations in *KRAS* were also located in hotspots. In *CTNNB1*, all mutations except one nonsense mutation were found in a hotspot region of less than 50 base pairs.

Although this study analyzed the full sequence of 101 genes, a cost-efficiency analysis was performed to determine whether limiting sequencing to *TP53*, *ARID1A*, and *PTEN*, while restricting *PIK3CA*, *KRAS*, and *CTNNB1* to hotspot regions, would still be effective for diagnostics. The analysis found that 92% of patients who tested positive using full-gene sequencing also showed positive results with this limited approach.

To further explore cost-saving measures, deep sequencing was conducted with an average depth of ×35,000 to achieve a cutoff of 0.05%. When the cutoff was raised to 0.1%, 99% of the positive results were still detected, with 91% remaining positive when using the partial sequencing strategy. Even at a higher cutoff of 0.25%—five times the original threshold—93% of the positive results were retained, with 86% still detectable using the combined whole-gene and hotspot sequencing approach. These findings suggest that focusing on key gene hotspots may help reduce costs for vaginal swab-based gDNA testing without significantly compromising sensitivity.

While vaginal swab-based gDNA testing is relatively expensive, this study examined cost-efficiency strategies. Despite the cost, it offers significant advantages over traditional dilation and curettage, including being faster, noninvasive, and not requiring anesthesia. The goal is to enable early detection of all gynecologic cancers. In cervical cancer, finding tumor DNA in vaginal swabs is trivially expected due to the anatomical location. However, early detection of ovarian cancer is challenging, leading to poor prognoses. Recently, vaginal swabs have emerged as a potential alternative for early detection, with ongoing biomarker as CA-125 and research on the application of NGS to vaginal swab-based gDNA from ovarian cancer patients(41, 42).

Detecting endometrial cancer through noninvasive methods has been explored in many studies, including urine, vaginal swabs, and menstrual blood sampling. These methods offer promising alternatives to invasive procedures, making early detection and regular monitoring more accessible. One method being investigated is urine sampling, where researchers focus on detecting DNA methylation markers(43). These studies report an area under the curve (AUC) ranging from 0.86 to 0.95, indicating a strong potential. However, sensitivity and specificity data were not reported, limiting the clinical utility of this approach. Additionally, the clinical use of blood-based DNA methylation tests is still limited because many institutions lack the necessary infrastructure for regular testing. As a result, this makes widespread implementation financially and logistically challenging. Moreover, DNA methylation testing cannot identify specific gene mutations and often requires additional sequencing for comprehensive analysis. Another study investigated the use of Pipelle, PAP-brush, and swab sampling for endometrial cancer detection through ultra-deep sequencing, achieving sensitivity rates of 81.9%, 55.2%, and 44.4%, respectively(44). However, in the control group of nonendometrial cancer patients, pathogenic mutations were detected in 37.2%, 33.1%, and 34.0% of cases. While the study suggested that these individuals were at higher risk, the relatively low sensitivity and specificity make these methods less viable for routine clinical use, especially with Pipelle sampling, which is not truly noninvasive. Another study on urine, cervicovaginal self-samples, and cervical scrapes for DNA methylation testing reported sensitivity from 0.89 to 0.93 and specificity from 0.90 to 0.92(45). These results demonstrate high potential for clinical application due to their favorable sensitivity and specificity. However, as previously mentioned, the current limitation of DNA methylation testing is that few institutions offer it for blood samples, and identifying specific gene mutations requires additional sequencing. Additionally, unlike NGS, which can accurately classify mutations using tier I and II guidelines, DNA methylation tests rely on AUC-based cutoffs and require both a cutoff-defining group and a test group for validation. These processes have not been fully addressed, indicating the need for further research to refine the approach. Menstrual blood has also been explored as a noninvasive sampling method for detecting HPV. with one study reporting a 94% concordance between menstrual blood-based and traditional HPV detection methods(46). However, 22.9% of the participants expressed discomfort with self-collection, and 94.0% preferred clinician-collected samples. Additionally, while the study showed that viral DNA can be detected, it has not yet progressed to detecting cancer-related mutations within the human genome, limiting its broader

application. In summary, while various noninvasive approaches show promise, they also come with significant limitations in sensitivity, specificity, and practicality for routine clinical use. This study, using a self-collectible vaginal swab-based gDNA approach combined with NGS, offers a more precise and reliable method for detecting key gene mutations, such as *PTEN* and *TP53*, that are directly associated with the prognosis of endometrial cancer. This self-collection method is user-friendly for patients and avoids the need for invasive procedures, making it a strong candidate for further research and potential clinical application.

Cervical, ovarian, and endometrial cancers differ not only anatomically and pathologically, but also in their mutational landscapes. Cervical cancer often has mutations in genes like *PIK3CA* and *PTEN*, similar to endometrial cancer, but it also frequently shows mutations in genes such as *EP300*, *FBXW7*, and *HLA-A*, which are less common in endometrial cancer(47). Additionally, chromosomal-level mutations, such as the commonly reported 3q amplification (occurring in 66% of cervical cancer cases), highlight the distinct mutational profiles of these cancers.

However, ovarian cancer has been the focus of extensive plasma-based ctDNA research, and it has been demonstrated that analyzing a limited set of genes, including *TP53*, *BRCA1*, *BRCA2*, *ARID1A*, *CCNE1*, *KRAS*, *MYC*, *PIK3CA*, and *PTEN*, provides sufficient diagnostic information(48). Thus, by targeting commonly mutated genes across all three cancers, a panel of 20 to 30 genes could be developed for analysis. This strategy could lead to a next-generation tool for early cancer detection that not only identifies the presence of cancer but also predicts the likely cancer type based on its mutational profiles.

When analyzing plasma-based ctDNA, its association with cancer stage was weaker than that of vaginal swab-based gDNA, but it showed a stronger correlation with lymphovascular invasion and prognosis. The weaker association with stage may be due to the fact that positive plasma-based ctDNA findings cannot entirely exclude clonal hematopoiesis of indeterminate potential(27).However, the strong correlation between plasma-based ctDNA and lymphovascular invasion and prognosis aligns with previous research suggesting that plasma-based ctDNA reflects tumor burden(49).

While the positive detection rate of plasma-based ctDNA was lower than that of vaginal swab-based gDNA, this study continued to research its potential as a prognostic variable due to its significant association with outcomes. In the Cox univariate regression analysis, plasma-based ctDNA, along with stage and lymphovascular invasion, had a highly significant p-value ($p<0.01$). In the multivariate Cox regression, both lymphovascular invasion and plasma-based ctDNA remained significant. Although stage did not reach statistical significance, raising concerns about possible overfitting, the current dataset was too small to confirm this, and the small number of patients with advanced-stage disease may have affected the result. Therefore, plasma-based ctDNA appears to be an effective tool for monitoring during follow-up and shows promise as a prognostic factor in cancer outcomes.

In previous studies, genes associated with endometrial cancer have been classified by molecular subgroups: *PTEN*, *FBXW7*, *ARID1A*, and *PIK3CA* are frequently mutated in the *POLE*-ultramutated group; *PTEN*, *PIK3CA*, *KRAS*, and *PIK3R1* in the MSI-hypermutated group; *PTEN*, *PIK3CA*, *CTNNB1*, and *ARID1A* in the copy number low group; and *TP53*, *PIK3CA*, and *PPP2R1A* in the copy number high group(9). Other studies have consistently identified *PTEN* and *PIK3CA* as the most frequently mutated genes in endometrial cancer, followed by *TP53*(50). However, in the plasma-based ctDNA analysis, *TP53* was identified as the most frequently mutated gene in all tiers of analysis.

The final analysis of plasma-based ctDNA suggests that its detection is related to tumor burden and is associated with poor prognosis(49). *PTEN* is often linked to better prognosis and lower cancer stages(13), while *TP53* is commonly associated with worse outcomes(14). Therefore, when assessing plasma-based ctDNA, which is correlated with tumor burden and unfavorable prognosis, *TP53* mutations may be detected more often.

Indeed, more than half of the patients with *TP53* mutations had advanced or recurrent disease, even though most cancer patients in this study were stage I. Although many patients achieved long-term disease-free survival, over half with detectable *TP53* mutations plasma-based ctDNA experienced recurrence or death. This high frequency of *TP53* mutations, along with their associated clinical outcomes, suggests that *TP53* detection should prompt more aggressive treatment and closer follow-up for these patients.

Recent research has increasingly focused on plasma-based ctDNA for predicting recurrence in advanced-stage cancers more rapidly(51). This is particularly true for ovarian cancer, where many patients present with advanced stages, and some studies suggest that plasma-based ctDNA may be a more efficient marker than CA-125(52, 53). In contrast, most endometrial cancer patients are diagnosed early(54), leading to lower recurrence rates and fewer studies on ctDNA in this context. Previous reports have indicated that endometrial cancer patients with negative plasma-based ctDNA results generally have longer progression-free survival and overall survival, which is consistent with findings(55). However, while that study only measured plasma-based ctDNA levels via PCR, this study utilized Cox multivariate regression to demonstrate that positive plasma-based ctDNA results independently impact prognosis.

Studies show that patients with high-risk endometrial cancer have higher plasma-based ctDNA levels than those with low-risk disease(56), supporting the claim that plasma-based ctDNA can monitor the tumor burden in these patients. These findings align with this study's premise and current views on the role of plasma-based ctDNA. While some reports suggest a correlation between plasma-based ctDNA and histopathological type, stage, and grade(57), others indicate no significant correlation(11), aside from stage and lymph node status, leading to some controversy. This study found that plasma-based ctDNA is associated with stage and lymphovascular invasion.

A small study with six patients reported that plasma-based ctDNA detected recurrence approximately 2.5 months earlier than conventional CT scans(58). In this study, plasma-based ctDNA also detected recurrence earlier than conventional imaging, but the number of recurrent cases followed up with serial plasma-based ctDNA measurements was very limited. Only about half of the cases were diagnosed at the same time as conventional methods, making it difficult to definitively conclude that plasma-based ctDNA is faster than conventional radiographic methods such as CT. Thus, further studies are needed, especially on patients with advanced-stage endometrial cancer who can be monitored with serial plasma-based ctDNA follow-up.

Molecular classification of endometrial cancer is essential for creating personalized treatment strategies that acknowledge the heterogeneity of the disease. Patients with MMR-D endometrial cancer have been shown to respond well to immunotherapy, expanding the spectrum of precision medicine in this cancer type(59). Recent studies, including this research, have shown that *TP53* mutations significantly impact the prognosis and aggressiveness of endometrial cancer. Patients with these mutations may benefit from standard chemotherapy and also from therapies targeting the p53 pathway, allowing for a more tailored approach(60). Also, since *TP53* mutation is strongly associated with poor prognosis, this study highlights the importance of early detection to adjust treatment strategies accordingly. Furthermore, *PIK3CA* and *PTEN* mutations indicate alterations in the PI3K/AKT/mTOR pathway, which regulates cancer cell growth and survival. For patients with these mutations, PI3K/mTOR inhibitors are promising therapeutic options alongside conventional treatments(61). The noninvasive genomic profiling techniques used in this study—vaginal swab-based gDNA and plasma-based ctDNA—enable early detection of these key mutations, improving the prospects for individualized treatment. Ultimately, such approaches enable patient-specific therapeutic interventions and plasma-based ctDNA monitoring to assess prognosis on a personalized basis, optimizing both treatment efficacy and long-term outcomes for endometrial cancer patients.

The aim of this study was to investigate whether vaginal swab-based gDNA could potentially help with diagnosis. Previous reports and this findings show that plasma-based ctDNA is currently the only method effective for analyzing recurrence, but its role is limited in early-stage endometrial cancer. This highlights the need to evaluate tissue-based NGS, and to examine the advantages and disadvantages of each testing method.

Tissue-based NGS analyzes the tumor directly, making it the standard due to its reliability and the absence of false positives. However, its invasive nature requires patient anesthesia, and it cannot differentiate between germline and somatic variants. In addition, tumor heterogeneity can pose limitations(10). In contrast, vaginal swab-based gDNA is a noninvasive test that allows for the easy assessment of the tumor’s genetic environment in many patients. Furthermore, with additional research, vaginal swabs could potentially be used to analyze both vaginal and ovarian cancers. However, they have a sensitivity of only 77.7%, meaning they may not detect all cases of

endometrial cancer. In this study, swabs were collected by a gynecologist under general anesthesia, but they can also be self-collected. Moreover, their use is limited in patients who have undergone cervical conization or hysterectomy, and, like tissue-based NGS, they cannot distinguish between germline and somatic variants. Plasma-based ctDNA; however, has the advantage of significantly impacting prognosis and allows for serial measurements to predict recurrence. Furthermore, it can detect germline variants by analyzing PBMCs from plasma. However, its sensitivity is low, particularly in low-stage cancers, and it is cannot completely exclude clonal hematopoiesis of intermediate potential, which poses a challenge.

Each test has its own strengths and limitations. If NGS becomes more affordable, an ideal strategy could be using vaginal swab-based gDNA for noninvasive screening, tissue-based NGS during surgery, and plasma-based ctDNA for prognosis and recurrence monitoring.

This study led to several key conclusions. First, vaginal swab-based gDNA shows potential as a less invasive alternative to tissue NGS for genomic profiling of endometrial cancer. Second, genomic profiling using vaginal swab-based gDNA, particularly focusing on genes such as *PTEN* and *TP53*, may aid in identifying prognostic factors, thereby enhancing the understanding of patient outcomes. Lastly, plasma-based ctDNA monitoring is promising for meaningful follow-up, providing insights into recurrence and prognosis, especially in higher-stage or recurrent cases, although further research is needed for its use in early-stage endometrial cancer. Together, these approaches offer a more comprehensive framework for both diagnosis and long-term monitoring of endometrial cancer patients.

The novel aspects of this study are as follows: It is the first to prospectively analyze a large cohort of patients using both vaginal swab-based gDNA and plasma-based ctDNA through deep sequencing, and unprecedented approach. This study also shifts from traditional classifications based on the MMR gene, *POLE*, and p53, aiming to streamline gene panel selection for genomic profiling. While serial plasma-based ctDNA monitoring has been explored in other cancers, this is the first large-scale application in endometrial cancer, advancing detection and prognostic evaluation in the field.

This study faced several limitations. First, being a prospective study, patient selection was limited, resulting in a small cohort of advanced-stage patients and few recurrences. While prognostic analysis was performed on the vaginal swab-based gDNA, particularly between the *PTEN* mutated group and both intact group, the small number of recurrent cases made it difficult to establish strong evidence supporting significant prognostic differences between these groups. Although a swimmer plot for recurrent patients was created based on plasma-based ctDNA, further analysis, such as assessing early diagnosis potential, was challenging due to the small sample size. Second, although this study successfully analyzed DNA mutations and copy number variations across 101 target genes, this study could not assess gene rearrangements or DNA methylation. Despite achieving deep sequencing with an average depth of over ×35,000 and demonstrating

feasibility at relatively higher VAF cutoffs, the inability to analyze structural variants or epigenetic changes was a limitation. Future research could address this by incorporating a broader range of genetic and epigenetic alterations. Third, not all patients underwent tissue-based NGS, which is not routinely performed in endometrial cancer treatment, limiting the study. Future studies should include all three methods—tissue-based NGS, vaginal swab-based gDNA, and plasma-based ctDNA—for better comparison. Finally, the study did not fully address variants from clonal hematopoiesis of intermediate potential. Currently, both vaginal swab-based gDNA and plasma-based ctDNA are expensive, and comprehensive PBMC analysis was financially and logistically challenging. Future studies should include comprehensive CHIP analysis, alongside genomic profiling of both germline and somatic variants, to improve plasma-based ctDNA accuracy.

V. Conclusion

In conclusion, this study demonstrates that vaginal swab-based gDNA testing is a promising noninvasive genomic profiling in endometrial cancer, helping to identify key mutations such as *PTEN* and *TP53*, which are important for early-stage prognosis. Plasma-based ctDNA also holds potential for monitoring recurrence and prognosis, particularly in advanced stages, although its use in early detection is limited. Although vaginal swab-based gDNA, tissue-based NGS, and plasma-based ctDNA each have distinct strengths and limitations, using them together could provide a comprehensive framework for the diagnosis, prognosis, and management of endometrial cancer. Further research is needed to refine these methods, improve their sensitivity, and evaluate their cost-effectiveness in broader clinical settings.

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Abstract in Korean

자궁내막암의 질 도말 및 혈장 샘플의 딥 시퀀싱 접근을 통한 유전체 특성 분석

자궁내막암은 미국에서 가장 흔한 부인암이며, 최근 한국에서도 자궁경부암을 제치고 가장 흔한 부인암으로 자리 잡았다. 이 질환의 이질성으로 인해 진단이 어려운 경우가 많으며, 현재의 조직 기반 차세대 염기서열 분석(NGS) 방법은 침습적이고 중요한 변이를 놓칠 가능성이 있다. 본 연구는 비침습적 대안으로 질 도말을 통한 유전체 DNA(gDNA)와 혈장 순환 종양 DNA(ctDNA)를 분석하여 자궁내막암의 유전체 프로파일링과 예후 예측 가능성을 평가하고자 했다.

본 연구는 자궁내막암 및 양성 질환을 포함한 총 191명의 환자를 대상으로 진행하였다. 각 환자에게서 질 도말 기반 gDNA와 혈장 기반 ctDNA를 수집하여 분석하였으며, *PTEN*, *TP53*, *PIK3CA*와 같은 변이를 포함한 101개 유전자에 대해 NGS를 실시했다. 고도기 환자에게는 수술 후 여러 시점에서 샘플을 수집하여, 혈장 기반 ctDNA가 재발 및 진행 감시에 유용한지 평가했다.

연구 결과, 질 도말 기반 gDNA는 77.7%의 민감도와 96.6%의 특이도를 보이며, 특히 초기 자궁내막암의 변이 검출에 유용함을 보였다. *PTEN*, *TP53*, *PIK3CA* 변이가 가장 흔했다. 이 연구는 새로운 분류 체계를 만들어 냈으며, *TP53* 변이는 나쁜 예후와 연관되어 있고, *PTEN* 변이는 좋은 예후와 연관되어 있는 것과 관련되어 있다. 또한, 질 도말 기반 gDNA는 림프관 침윤과 같은 예후 인자와 강한 상관관계를 보였으며, 음성 세포진 검사에서도 전통적인 PAP 세포진보다 암 검출에 효과적이었다.

혈장 기반 ctDNA 분석은 초기 암에서는 민감도가 낮았으나, 고도기 질환, 림프관 침윤 및 재발과 밀접하게 관련되어, 치료 후 모니터링에 잠재적 유용성을 보였다. Cox 회귀 분석을 통해 림프관 침윤 및 혈장 기반 ctDNA 양성 결과가 중요한 예후 인자임을 확인했으며, 혈장 기반 ctDNA 양성 환자는 결과가 더 나빴으며 일부 경우에는 기존 영상 검사보다 재발을 더 일찍 예측할 수 있음을 시사했다.

본 연구의 독창적인 점은 대규모 자궁내막암 환자 집단에서 질 도말 기반 gDNA와

혈장 기반 ctDNA를 딥 시퀀싱을 통해 분석한 점이다. 이러한 비침습적 기법을 통해 종양 진행 및 저항성에 관한 유전체 변화를 시간 경과에 따라 추적할 수 있었으며, 3가지 유전자의 모든 부분과 3가지 유전자의 핫스팟 부분을 판독하는 방식으로 검사 비용을 낮추면서도 높은 진단 정확도를 유지할 수 있는 효율적인 대안을 모색했다.

결론적으로, 질 도말 기반 gDNA는 자궁내막암의 진단 및 유전체 프로파일링에 유망한 방법이며, 주요 예후 변이를 식별하는 데 효과적이다. 혈장 기반 ctDNA는 자궁내막암에서 종양 부담, 재발 및 예후에 대한 중요한 정보를 제공하여 이를 보완한다. 이 두 방법을 결합하면 자궁내막암의 진단과 모니터링을 개선할 수 있으나, 정확성 향상, 임상적 활용의 정교화, 민감도 최적화 및 폭넓은 임상 적용에 대한 비용 효과 평가를 위한 추가 연구가 필요하다.

핵심되는 말 : 자궁내막암, 질 도말 기반 gDNA, 혈장 기반 ctDNA, 차세대 염기서열 분석, *TP53* 돌연변이, *PTEN* 돌연변이, 예후 인자, 재발 모니터링, 비침습적 진단

PUBLICATION LIST

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