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Original Article

Clinical evaluation of a droplet digital PCR assay for detecting *POLE* mutations and molecular classification of endometrial cancer

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ABSTRACT

Objective: We evaluated droplet digital polymerase chain reaction (ddPCR) method for detecting *POLE* mutations in endometrial cancer (EC) and guiding its molecular classification.
Methods: We reviewed 240 EC specimens from our hospital database. A ddPCR assay was used to identify *POLE* mutations at 5 known hotspots (P286R, S297F, V411L, A456P, and S459F). Expressions of p53 and mismatch repair proteins were identified using immunohistochemistry.
Results: The ddPCR assay identified *POLE* mutations in 10.8% of patients. The most common mutation was V411L (61.54%), followed by P286R (23.07%), S459F (7.69%), S297F (3.85%), and A456P (3.85%). Eight/one cases had positive ddPCR but negative Sanger sequencing/next-generation sequencing, respectively. Molecular classification revealed *p53*-mutated subtype as significantly more common for tumors with a high International Federation of Gynecology and Obstetrics (FIGO) grade, deep myometrial invasion, lymphovascular space invasion, advanced stage, and high/advanced risk groups; the *POLE* mutated group was more frequent in the low stage and low/intermediate risk group. Survival analyses revealed the poorest outcomes for *p53*-mutated EC, while mismatch repair-deficient and no specific molecular profile ECs had similar progression-free survival (PFS) outcomes, and *POLE*-mutated ECs had the best PFS outcome ($p<0.001$). When only intermediate, high-intermediate, and high-risk groups were analyzed for subgroups, molecular classification still showed differences both in PFS ($p=0.003$) and overall survival ($p=0.017$).
Conclusion: Hotspot *POLE* mutations can be detected using the ddPCR assay. We suggest simultaneously evaluating *POLE* mutation status using ddPCR and p53/mismatch repair protein expressions using immunohistochemistry, which can rapidly and accurately determine the molecular subtype of EC.

Keywords: Polymerase Chain Reaction; *POLE*; Classification; Endometrial Cancer; Prognosis

Synopsis

Using droplet digital polymerase chain reaction (ddPCR), we successfully detected the 5 most frequent pathogenic hotspot mutations in the *POLE* gene in samples from endometrial cancer patients, thus confirming the utility of ddPCR in their identification.

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Presentation
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Conflict of Interest
No potential conflict of interest relevant to this article was reported.

Author Contributions
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INTRODUCTION

Endometrial cancer (EC) is the second most common gynecologic cancer in the developed countries [1] with increasing rates of incidence over the past decades even in developing countries. Most women, especially those with low-grade early-stage EC, experience excellent outcomes. Although some advanced and high-risk patients show high recurrence and low survival rates, survival is expected to improve with either radiation therapy or chemoradiation in such patients [2,3]. Therefore, accurate risk stratification is necessary in order to determine the eligibility of EC patients for adjuvant treatment.

The Cancer Genome Atlas (TCGA) Research Network has defined 4 subgroups of EC [4], and the molecular endometrial classification system using surrogate markers such as Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE) system was subsequently developed [5-7]. Subsequently, the World Health Organization (WHO) confirmed 4 molecular subgroups of EC that were correlated with prognosis [8]. The *POLE*-mutated subtype is characterized by mutations in the exonuclease domain of polymerase-ε (POLE) and has an excellent prognosis. The mismatch repair-deficient (MMR-D) subtype is characterized by microsatellite instability with deficient expression of MMR proteins and has an intermediate prognosis. The *p53*-mutated subtype is characterized by a high copy number alteration rate, *TP53* mutations with aberrant p53 expression, and a poor prognosis. The no specific molecular profile (NSMP) subtype has an intermediate prognosis. Immunohistochemistry may be used as a surrogate for molecular sequencing, given that it is easier to perform, more commonly used in practice, and less expensive [9,10].

Although MMR and p53 status can be easily detectable via immunohistochemical staining, there is currently no standard method for identifying *POLE* mutations, which are generally identified using next-generation sequencing (NGS) or Sanger sequencing [5,11-15]. However, with the exception of *POLE*, there are few EC-related genes that are targetable during NGS, which is expensive and requires a prolonged turn-around time. Sanger sequencing is useful for evaluating exons 9, 11, 13, and 14 of *POLE*, where mutation hotspots are concentrated, although it has a low limit of detection and there is a strong possibility of false-negative results for mutations with a low frequency. In ECs, the most frequent somatic mutations in the proofreading domain of *POLE* are P286R, V411L, and S459F [11], which are identified in 67%–92% of *POLE*-mutated cancers [5,10,11]. It might be preferable to sequence the entire exonuclease domain, although clinical application would likely require focusing on known pathogenic *POLE* variants. León-Castillo et al. [16] recently reported that P286R, V411L, S297F, A456P, and S459F were “hotspot” *POLE* mutations, and the revised WHO classification system for EC recommends hotspot analysis that can detect these 5 mutations, along with NGS and Sanger sequencing [8].

Droplet digital polymerase chain reaction (ddPCR) has been developed [17] as a quantitative method for detecting even small amounts of genetic variation [18]. There is increasing interest in using ddPCR to detect rare mutations, copy number variations, and gene rearrangements [19-23]. Furthermore, ddPCR can be performed using paraffin-embedded specimens and has a turn-around time of 1–2 days, which makes it an attractive diagnostic test. Therefore, we evaluated whether ddPCR could accurately detect *POLE* mutations in EC specimens, and whether the ddPCR results could be combined with immunohistochemical MMR/p53 protein expression for molecular classification of EC.

MATERIALS AND METHODS

1. Patients and samples

We reviewed medical records and pathological reports for 240 patients with EC who had undergone surgery at our hospital between May 2006 and January 2013. Data regarding age at diagnosis, depth of invasion, histological subtype, International Federation of Gynecology and Obstetrics (FIGO) grade, FIGO stage, and treatment modality were extracted. Hematoxylin and eosin-stained tumor sections were reviewed by 2 gynecological pathologists to confirm the diagnosis based on the 2020 WHO classification system [8]. Tumors were included if they involved endometrioid, serous, clear cell, or mixed histology, while carcinosarcoma was excluded. None of the patients had received neoadjuvant therapy. The retrospective protocol was approved by the institutional review board of Seoul National University Bundang Hospital, which waived the requirement for informed consent (B-2008/628-304).

2. Using ddPCR to detect POLE mutations

The ddPCR was performed using a ddPCR system (Qx200 Droplet Digital™ PCR System; Bio-Rad, Hercules, CA, USA) as per the manufacturer's recommended protocol. The details of the specific mutations are provided in **Table S1**.

Briefly, 8.3 μL Droplex POLE mixture was mixed with 11.7 μL genomic DNA from patients to a final reaction volume of 20 μL in a PCR tube strip of eight 0.2-mL conical-bottom PCR tubes (Axygen, Tewksbury, MA, USA). Subsequently, 20 μL of reaction mixture was loaded into each well of DG8TM Cartridge (Bio-Rad) followed by 70 μL of Droplet Generation Oil for Probes (Bio-Rad) into each corresponding well. Droplets were generated using Droplet Generator (Bio-Rad) following the manufacturer's instructions. A collection of uniformly sized aqueous droplets was produced in the monodisperse water-in-oil emulsion format. The resulting emulsion was transferred to a 96-well PCR plate (Eppendorf, Hamburg, Germany or Bio-Rad) by pipetting. The plate was sealed with pierceable foil heat seal using PCR plate sealer (Bio-Rad) and placed into a thermal cycler with a hot lid (Proflex PCR system; Life Technologies, Carlsbad, CA, USA). The thermocycling conditions were as follows: one cycle of 30 minutes at 37°C, followed by 10 minutes at 95°C; 40 cycles of 30 seconds at 94°C; 1 minutes at 60°C, and a final step at 98°C for 10 minutes, with the ramp rate settled at 2°C/sec. After thermal cycling, the plate was loaded to measure the endpoint fluorescence signal from each droplet using Qx200 Droplet Reader (Bio-Rad). The droplet reader connected to a laptop computer running data analysis QuantaSoft software (v1.6.6.0320; Bio-Rad) and run data analysis was carried out as followed. Each individual droplet was defined on the basis of the fluorescent amplitude as being either positive or negative. Threshold was determined manually based on the amplitude of positive control well containing wild-type genomic DNA and standard positive DNA. The number of positive and negative droplets were distinguished by threshold, and the given numbers were used for calculating the concentration target in terms of copies/μL. The threshold values of P286R, S297F, V411L, A456P, and S459F were (3000), (3500), (2800), (3200) and (3400), respectively, which were adjusted according to each test condition. According to the fluorescent signal of the negative control, the cut-off for *POLE* mutation was ≥6 copies/20 μL or a mutation index (MI, %, mutant copies for each mutation/the mutant copies of the internal control) ≥0.3%.

3. Sanger sequencing

All samples were independently validated using Sanger sequencing in a double-blind fashion, and the results were matched after analysis. The sequences of the primers used in Sanger

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sequencing are stated in **Table S2**. Sanger sequencing was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions, and the products were analyzed using an ABI3730XL system (Thermo Fisher Scientific). All variants were described according to the LRG_789 reference sequence (NM_006231.3) and the Human Genome Variation Society guidelines.

4. Targeted sequencing and tumor mutation burden (TMB) calculation

We performed targeted NGS on 11 cases with a *POLE* mutated case with a low MI of 5% or less in the ddPCR discrepancy between Sanger sequencing and ddPCR. DNA isolated from formalin-fixed, paraffin-embedded (FFPE) tumor sections were subjected to hybridization capture-based NGS to detect somatic alterations in *POLE* gene with a 1.67-Mbp custom capture probe (SureSelect DNA Advanced Design Wizard, Agilent, Santa Clara, CA, USA), as previously described [24]. Reads were aligned to the genome build GRCH19 using BWA, followed by the removal of duplicates using Picard Tools, indel realignment, and base recalibration using the Genome Analysis Toolkit (GATK; Broad Institute, Cambridge, MA, USA). Somatic mutation calling was performed using MuTect2. TMB, defined as the number of nonsynonymous and in-frame shift mutations per megabase (Mb), was also calculated.

5. Tissue microarray construction and immunohistochemistry for p53 and MMR proteins

Tumor areas were marked in the tissue sections from each patient. To minimize the effects of protein expression heterogeneity, paired 2-mm core tumor samples were extracted from each donor block and arranged in a new tissue microarray (TMA) block using a trephine apparatus (SuperBioChips Laboratories, Seoul, Korea).

Immunohistochemistry was performed using the TMA to evaluate p53 and MMR protein expressions (hMLH1, hMSH2, hMSH6, and PMS2). Staining for p53 was performed using a primary monoclonal antibody (pre-diluted DO-7; Dako, Santa Clara, CA, USA) as previously described [25], and expression was considered aberrant if >75% of the cells were strongly positive for p53 (overexpressed) or 0% of the cells were positive (the null phenotype). Staining for the MMR proteins was performed using primary monoclonal antibodies against MLH1 (G168-728, 1:250; PharMingen, San Diego, CA, USA), MSH2 (FE11, 1:50; Oncogene Research Products, Cambridge, MA, USA), MSH6 (GRBP.P1/2.D4, 1:200; Serotec Inc, Raleigh, NC, USA), and PMS2 (A16-4, 1:200; PharMingen). Expression was defined as abnormal based on the complete absence of one or more of the MMR proteins from all tumor cell nuclei [26].

6. Statistical analysis

The relationships between molecular subgroups and clinicodemographic characteristics were evaluated using the χ^2 test and analysis of variance, as appropriate. Survival curves were created using the Kaplan-Meier method and compared using the log-rank test. All analyses were 2-sided and significance was set at $p<0.05$. All analyses were performed using IBM SPSS software (version 25.0; IBM Corp., Armonk, NY, USA).

RESULTS

1. Patient characteristics

Table 1 shows the clinicopathological characteristics of the 240 patients. The mean age at diagnosis was 55.75 years (range: 27–83 years). The histological subtypes were defined as type

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Table 1. Patient demographics

Characteristics	Total
Age (yr)	55.75 (27–83)
Histological subtype	
Type I	212 (88.3)
Type II and mixed	28 (11.7)
FIGO grade	
Low (1–2)	185 (77.1)
High (3)	55 (22.9)
Myometrial invasion	
<1/2	163 (67.9)
≥1/2	77 (32.1)
LVSI	
Absent	161 (67.1)
Present	79 (32.9)
FIGO stage	
I	198 (82.5)
II	5 (2.1)
III	30 (12.5)
IV	7 (2.9)
ESMO-ESGO-ESTRO risk group	
Low	108 (45)
Intermediate	41 (17.1)
High-intermediate	27 (11.3)
High	57 (23.7)
Advanced	7 (2.9)
Adjuvant treatment	
None	129 (53.8)
Radiotherapy alone	39 (16.3)
Chemotherapy alone	37 (15.4)
Chemoradiotherapy	35 (14.6)
Total	240 (100)

Values are presented as mean (range) or number (%).
ESMO-ESGO-ESTRO, European Society for Medical Oncology-European Society of Gynaecological Oncology-European Society for Radiotherapy & Oncology; FIGO, International Federation of Gynecology and Obstetrics; LVSI, lymphovascular space invasion.

I (pure endometrioid) (212/240 patients, 88.3%) and type II (16 serous and 4 clear cell) or mixed (28/240 patients, 11.7%). The FIGO stages at diagnosis were stage I (198/240 patients, 82.5%), stage II (5/240 patients, 2.1%), stage III (30/240 patients, 12.5%), and stage IV (7/240 patients, 2.9%).

Patients were classified into low, intermediate, high-intermediate, high, and advanced risk groups according to the European Society for Medical Oncology (ESMO), European Society for Radiotherapy & Oncology (ESTRO) and European Society of Gynaecological Oncology (ESGO) consensus guideline [27]. In total, 111 patients (46.3%) had received adjuvant therapy (radiotherapy: 39 patients [16.3%], chemotherapy: 37 patients [15.4%], chemoradiotherapy: 35 patients [14.6%]). The mean follow-up period was 67.4 months (range: 0.6–170.7 months). During follow-up, 16.7% of the patients experienced recurrence and 6.3% of patients died because of their disease. The median progression-free survival (PFS) was 67.4 months (range: 0.6–170.7 months) and the median overall survival (OS) was 72.1 months (range: 3.4–170.7 months).

2. Detection of POLE mutations and clinicopathological features

The ddPCR identified *POLE* mutations in 26 patients (10.8%). The mean mutant copies for *POLE*-mutated cases were 509.65 (range: 18.8–2,800) and the mean MI was 10.8% (range: 0.8%–

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Table 2. Comparing the *POLE* results from ddPCR and Sanger sequencing according to mutation site

Methods	Mutation														
	P286R			S297F			V411L			A456P			A459F		
	No.	ddPCR copies	Sanger	No.	ddPCR copies	Sanger	No.	ddPCR copies	Sanger	No.	ddPCR copies	Sanger	No.	ddPCR copies	Sanger
Results	1	1,170 (25.4)	C > G	7	872 (29.9)	C > T	8	2,800 (17.9)	G > T	24*	42 (2.1)	WT	25	1784 (24.8)	C > T
	2	1,296 (42.6)	C > G				9	1,028 (16.6)	G > T				26*	44 (1.7)	WT
	3	682 (12.4)	C > G				10	640 (11.9)	G > C						
	4	104 (10.2)	C > G				11	530 (12.4)	G > T						
	5*	72 (3.4)	C > G				12*	410 (6.6)	WT						
	6*	44 (1.5)	WT				13*	342 (4.8)	G > T						
							14	282 (12.4)	G > C						
							15	240 (7.7)	G > T						
							16	228 (8.1)	G > T/C						
							17*	194 (3.8)	WT						
							18	128 (10.2)	G > T						
							19*	112 (4.7)	G > T						
							20	96 (5.7)	G > T						
							21*	48 (1.7)	WT						
							22*	44 (0.8)	WT						
							23*	18.8 (1.6)	WT						
Mutation detection agreement		Reference	83.30%		Reference	100%		Reference	68.75%		Reference	0		Reference	50%

Values are presented as mutation index (%).
ddPCR, droplet digital polymerase chain reaction; RT-PCR, real-time polymerase chain reaction; WT, wild type.
*Cases performed targeted sequencing.

42.6%). The most common mutation was V411L (61.54%, 16/26), which was followed by P286R (23.07%, 6/26), S459F (7.69%, 2/26), S297F (3.85%, 1/26), and A456P (3.85%, 1/26) (**Table 2**).

There were 8 cases with positive ddPCR results but negative Sanger sequencing results, which involved V411L (5 cases), P286R (1 case), A456P (1 case), and S459F (1 case). Majority of cases (6/8) had a low CN (<50) and a MI of <2.2% (**Table 2**).

3. Correlation between NGS and ddPCR results

We analyzed the *POLE* mutation and TMB by targeted sequencing in 8 cases showing discrepancy between ddPCR and Sanger sequencing and 3 cases showing a low MI value of less than 5% in ddPCR even if detected in Sanger sequencing 9 (**Table 2**). In 10 of 11 cases, *POLE* mutations identical to those of ddPCR were observed by targeted sequencing. The discrepancy was a case in which a very low copy of V411L mutation was observed in ddPCR (case number 22 in **Table 2**). TMB ranged from 72.5/Mb in the lowest case to 1,991.7/Mb in the highest case, and the average TMB was 613.4/Mb.

4. Molecular classification using surrogate markers

We performed molecular classification using the surrogate markers mentioned above. Three cases were assigned 2 molecular subtypes simultaneously (1 case of *POLE*-mutated and MMR-D, 2 cases of MMR-D and *p53*-mutated) and 2 cases were assigned 3 subtypes simultaneously. Those cases were classified into one of the 4 groups based on the on the priority of presence of *POLE* mutation > MMR-D > *p53* mutation; 3 cases in which *POLE* mutations were observed were classified as *POLE* mutated subtypes, and 2 cases in which MMR-D and *p53* mutations were simultaneously observed were classified as MMR-D subtypes [28,29].

Subtype-related differences were observed in the clinicopathological characteristics (**Table 3**). The type I and type II/mixed subgroups exhibited marked differences in their molecular subtypes (p<0.001). Type I EC most commonly involved the NSMP subtype (58.0%, 123/240),

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Table 3. Clinicopathological features according to molecular classification

Characteristics	*POLE*-mut	MMR-D	*p53*-mut	NSMP	p-value
Age (yr)	56.12 (39–76)	53.81 (30–70)	60.59 (27–78)	54.84 (30–83)	>0.050
Histological subtype					
Type I	26 (12.3)	46 (21.7)	17 (8.0)	123 (58.0)	<0.001
Type II and mixed	0	1 (3.6)	24 (85.7)	3 (10.7)	
FIGO grade					
Low (1–2)	22 (11.9)	37 (20.0)	11 (5.9)	115 (62.2)	<0.001
High (3)	4 (7.3)	10 (18.2)	30 (54.5)	11 (20.0)	
Myometrial invasion					
<1/2	18 (11.0)	30 (18.4)	20 (12.3)	95 (58.3)	0.015
≥1/2	8 (10.4)	17 (22.1)	21 (27.3)	31 (40.3)	
LVSI					
Absent	16 (9.9)	29 (18.0)	20 (12.4)	96 (59.7)	0.008
Present	10 (12.7)	18 (22.8)	21 (26.5)	30 (38.0)	
FIGO stage					
Early (I–II)	23 (11.3)	37 (18.2)	27 (13.3)	116 (57.1)	<0.001
Advanced (III–IV)	3 (8.1)	10 (27.0)	14 (37.8)	10 (27.0)	
ESMO-ESGO-ESTRO risk group					
Low	11 (10.2)	29 (17.6)	4 (3.7)	74 (68.5)	<0.001
Intermediate	7 (17.1)	9 (22.0)	3 (7.3)	22 (53.7)	
High-intermediate	5 (18.5)	7 (25.9)	3 (11.1)	12 (44.4)	
High	2 (3.5)	12 (21.1)	28 (49.1)	15 (26.3)	
Advanced	1 (14.3)	0	3 (42.9)	3 (42.9)	
Adjuvant treatment					
None	13 (10.1)	22 (17.1)	9 (7.0)	85 (65.9)	<0.001
Radiotherapy alone	6 (16.2)	1 (2.7)	19 (51.4)	11 (29.7)	
Chemotherapy alone	4 (10.3)	10 (25.6)	7 (17.9)	18 (46.2)	
Chemoradiotherapy	3 (8.6)	14 (40.0)	6 (17.1)	12 (34.3)	
Total	26 (10.8)	47 (19.6)	41 (17.1)	126 (52.5)	

Values are presented as mean (range) or number (%).
ESMO-ESGO-ESTRO, European Society for Medical Oncology-European Society of Gynaecological Oncology-European Society for Radiotherapy & Oncology; FIGO, International Federation of Gynecology and Obstetrics; LVSI, lymphovascular space invasion; MMR-D, mismatch repair-deficient; p53-mut, p53-mutated; NSMP, no specific molecular profile; POLE-mut, DNA polymerase epsilon-mutated.

which was followed by the MMR-D (21.7%, 46/240), *POLE*-mutated (12.3%, 26/240), and *p53*-mutated (8.0%, 17/240) subtypes. In contrast, type II/mixed ECs most commonly involved the *p53*-mutated subtype (85.7%, 24/28) and only a few cases involved the NSMP (10.7%, 3/28) and MMR-D (3.6%, 1/28) subtypes. The *p53*-mutated subtype was significantly more common for tumors with a high FIGO grade, deep myometrial invasion, lymphovascular space invasion, advanced stage and high/advanced risk groups, whereas the *POLE* mutated group was more frequent in the low stage and low/intermediate risk group (**Table 3**).

5. Survival analysis and molecular testing algorithm

Among all patients, the PFS rate was 83.3% (200/240 patients) and the OS rate was 93.7% (225/240 patients). Survival outcomes were associated with molecular classification (PFS: p<0.001, OS: p<0.001), subtype (PFS: p<0.001, OS: p<0.001), tumor grade (PFS: p<0.001, OS: p<0.001), depth of invasion (PFS: p<0.001, OS: p=0.003), lymphovascular space invasion (PFS: p=0.002), tumor stage (PFS: p<0.001), risk stratification (PFS: p<0.001, OS: p=0.001), and adjuvant treatment (PFS: p<0.001, OS: p=0.007). The poorest PFS outcomes were observed for *p53*-mutated ECs, while MMR-D and NSMP ECs had similar PFS outcomes, and *POLE*-mutated ECs had the best PFS outcomes (**Fig. 1A**). The poorest OS outcomes were observed for *p53*-mutated ECs, NSMP ECs had moderate OS outcomes, and MMR-D and *POLE*-mutated ECs had similar OS outcomes (**Fig. 1B**). When only intermediate, high-intermediate, and high-risk groups were analyzed for subgroups, molecular classification still showed differences between PFS and OS (**Fig. 1C and D**).

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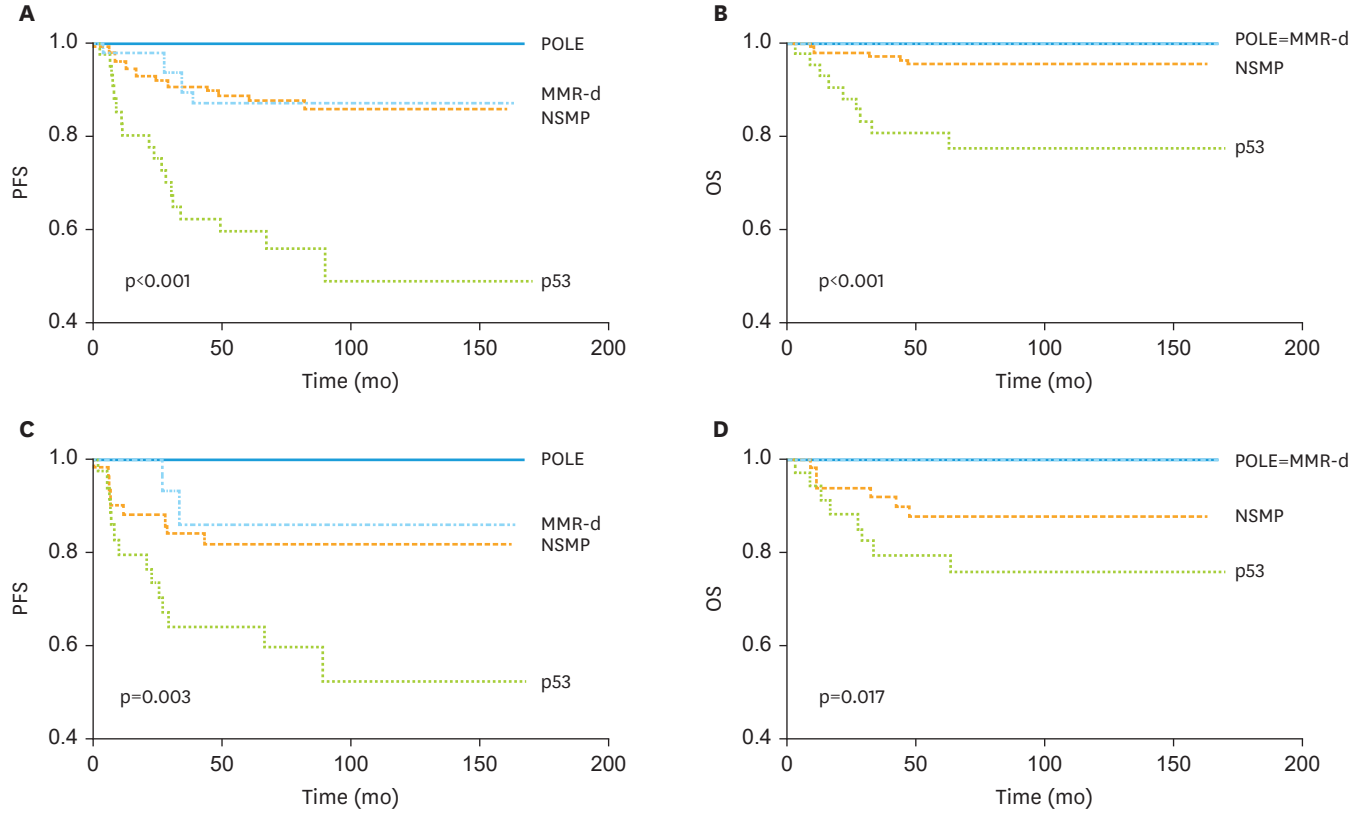

Fig. 1. Survival analysis for the 4 molecular classes of endometrial cancer. Kaplan-Meier curves are shown for (A, C) progression-free survival (PFS) and (B, D) overall survival (OS) for the entire cohort and for only intermediate, high-intermediate and high-risk groups. The p-values were calculated using the log-rank test.
MMR-D, mismatch repair-deficient; NSMP, no specific molecular profile.

Based on these results, we propose a molecular testing algorithm (**Fig. 2**) using immunohistochemical staining of five 4-μm sections to determine p53/MMR protein expressions and simultaneous ddPCR testing of five 8-μm sections to determine *POLE*

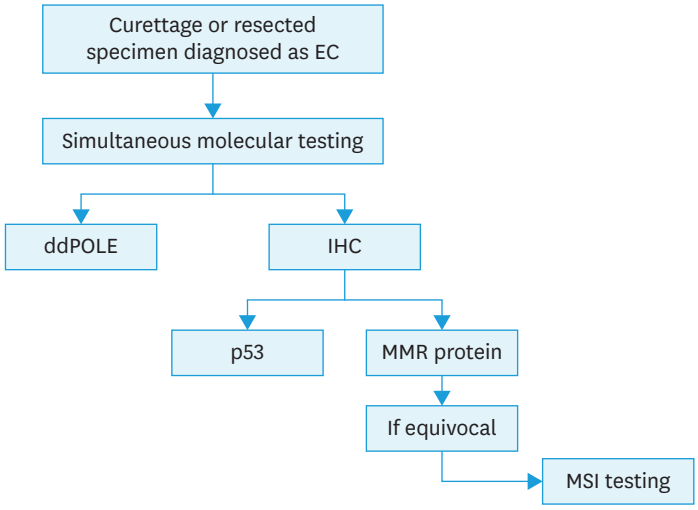

Fig. 2. Proposed simultaneous molecular testing algorithm for endometrial cancer (EC) patients. Five 4-μm section slides are subjected to immunohistochemistry (IHC) for p53 and mismatch repair proteins, and five 8-μm section slides are prepared and simultaneously subjected to digital droplet POLE (ddPOLE) testing. This strategy provides rapid and accurate determination of the molecular subtype, even for small biopsy or curettage specimens.

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mutation status. This protocol might provide a rapid and accurate determination of molecular subtype, even for EC patients with small biopsy or curettage specimens.

DISCUSSION

In this study, we successfully detected the 5 most frequent pathogenic hotspot mutations in the *POLE* gene in FFPE samples from EC patients using ddPCR. In addition, the potential of molecular classification performed by integrating the immunohistochemistry results of p53 and MMR proteins as surrogate markers was confirmed in accurately predicting the prognosis of EC patients. To the best of our knowledge, this is the first study to detect hotspot *POLE* mutations in EC patients via ddPCR.

The frequencies of *POLE* mutations detected using ddPCR were 10.8%, which are similar to previously reported frequencies (5%–12%) [5-7,30-32]. About 30% of the *POLE* mutations detected by ddPCR (8/26) were not detected by Sanger sequencing; it is considered that *POLE* mutations with low mutation allele frequency are not detected due to the limit of detection in Sanger sequencing. In fact, most of the mutations not detected by Sanger sequencing showed very low copy numbers of mutant alleles as detected by ddPCR, and those were detected in NGS. Although the ddPCR protocol in our study could not detect all *POLE* mutations and did not include all 11 known pathogenic mutations [16], these 5 mutations account for 84% of the 11 pathogenic mutations [16], and because ddPCR can detect mutations that appear infrequently, the ddPCR *POLE* hotspot test may be useful in detecting effective *POLE* mutations and predicting the prognosis of EC patients.

Interestingly, the most common ddPCR-detected mutations were V411L (67%) and P286R (25%), while analysis of TCGA data revealed that P286R was more common than V411L [4,16]. The high frequency of the V411L mutation in our study has 3 possible explanations. First, 6 of 16 cases with the V411L mutation (37.5%) had an MI of <5%, which may suggest that the variant allele frequency of V411L is low (vs. other pathogenic *POLE* variants), which would lead to a lower detection rate using NGS or Sanger sequencing. Second, all 3 cases that simultaneously involved the MMR-D and *p53*-mutated subgroups had the V411L mutation, which might suggest a relationship with MMR-D and genomic instability [28]. Third, there is a possibility of ethnic differences in the incidence of *POLE* variants. Thus, large-scale studies are needed to clarify the significance of *POLE* variants.

We performed molecular classification by integrating the *POLE* mutations detected by ddPCR and expression of p53 and MMR proteins confirmed by immunohistochemistry. Compared with the study of Talhouk et al. [5], our cohort showed a similar fraction of *POLE* mutated and p53-abnormal groups; however, the proportion of the MMR-D group was small and that of the NSMP group was high. This could likely be due to the evaluation of MMR immunohistochemistry in TMA. Unlike in the whole slide, wherein subclonal loss of MMR protein can be accurately evaluated, the evaluation in TMA is limited, which may have influenced the good prognosis of the MMR-D group in OS. In future, we aim to conduct a molecular classification study supplementing the MMR immunohistochemistry analysis method in a larger cohort of patients with EC.

We believe that using ddPCR to detect *POLE* hotspot mutations can complement the existing ProMisE classification system, which involves immunohistochemistry for evaluating MMR

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status, followed by *POLE* mutation testing and immunohistochemistry for evaluating p53 expression [5]. Murali et al. [29] also recently suggested that *POLE* sequencing should be performed before the immunohistochemistry for MMR status, as a negative result for MMR proteins in a *POLE*-mutated case would be assigned to the MMR-D subgroup, rather than the *POLE*-mutated subgroup. Our proposed algorithm involves simultaneous immunohistochemistry and ddPCR to evaluate *POLE*, MMR, and p53 statuses, which could prevent misclassification and provide a rapid turn-around time. This information could then be used for prognostication and treatment selection.

This study is limited by the small single-center retrospective design. Thus, a large number of cases should be accumulated in a prospective study to validate ddPCR detection of *POLE* mutation status, as well as the utility of our proposed algorithm.

In conclusion, we confirmed that ddPCR could detect hotspot *POLE* mutations in EC specimens. Moreover, we proposed a testing algorithm that involves simultaneous ddPCR to determine *POLE* status and immunohistochemistry to determine p53/MMR status, which could rapidly and accurately determine the molecular subtype of EC.

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SUPPLEMENTARY MATERIALS

Table S1

Mutation coverage of the GenesWell ddPOLE mutation kit

Click here to view

Table S2

Primer sequences for Sanger sequencing

Click here to view

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